LATE RADIATION MORBIDITY: INCIDENCE IN A SOUTH-EAST SCOTTISH COHORT AND INVESTIGATION INTO ABNORMALITIES IN DNA DOUBLE-STRAND BREAK REPAIR AND DAMAGE RESPONSE

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Abstract

Late normal tissue injury is dose-limiting for radiation therapy of cancer. The molecular mechanisms of this injury are unknown. However, almost all radiosensitive animals and cell lines are deficient in some aspect of DNA repair. We have derived EBV-transformed cell lines from five patients with late radiation injury to determine whether there was any evidence of reduced activity or expression of the enzymes active in non-homologous recombination, the major mammalian repair pathway for DNA double-strand breaks which cause radiation-induced cell death. Two of these cell lines exhibit post-radiation viability intermediate between normal controls and a cell line from an individual with ataxia-telangiectasia. DNA-dependent protein kinase activity *in vitro* was reduced 8-10-fold in these two cell lines compared to normal controls. The primary tumours from one of these patients, and a post-radiation cervix biopsy form the second, exhibited no immunoreactivity with a polyclonal antibody against the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs). Immunoblotting showed normal levels of Ku70, Ku80 and XRCC4, and the presence of DNA-PKcs, in both cell lines. This suggests that the DNA-dependent protein kinase might be an important factor in determining the predisposition of radiotherapy patients to late radiation injury.

ABBREVIATIONS

AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride
ATP	Adenosine triphosphate
dNTP	Nucleotides
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetra acetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
$MgCl_2$	Magnesium chloride
PBS	Phosphate Buffered Saline
RPMI	Roswell Park Memorial Institute
TE	Tris-EDTA
Tris-HCl	Tris (hydroxymethyl) aminomethane Hydrochloride

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CHAPTER 1

INTRODUCTION

1.1 Radiation treatment is an integral part of cancer therapies

Cancer is the leading cause of death in many parts of the world; in 2000, an estimated 6 million people died from the disease globally, with an expected increase in cancer deaths to 10 million per year by 2020 (World Cancer Report, World Health Organisation, 2001). 50 to 60% of patients with cancer are projected to require radiation treatment in the course of their battle against this disease (International Atomic Energy Agency, 2003).

1.2 Late Radiation Injury to Normal Tissue Limits the Radiation Dose

For individuals undergoing a curative course of radiotherapy, up to about 5% are estimated to experience an unexpected, severe late morbidity (Busch D, 1994). Severe morbidity is usually defined as grade 4 morbidity according to the late morbidity score defined by the Radiation Therapy Oncology Group (RTOG) and the European Organisation for Research and Treatment of Cancer (EORTC). Late morbidity is defined as morbidity arising more than 90 days after the start radiation treatment. Late morbidity caused by radiation is generally irreversible and has a potentially devastating effect on a patient's quality of life.

By limiting tumour doses to that which results in severe late morbidity in no more than 5% of the patient population treated, local control in some cancers is achieved in only a fraction of the total number of patients treated (Kagan AR, 1988).

Analysis of dose-response curves suggests that the injured patients are not a random sample of the total patient population but instead constitute a radiation-sensitive subset (Kagan AR, 1988). This is supported by the work of Turesson *et al.* in which it was demonstrated that as much as 80% of the variation in normal tissue

reactions could not be accounted for by known factors, and thus the variation was likely due to differences in intrinsic cellular radiosensitivity (Turesson I *et al.,* 1996; Safwat A *et al.,* 2002).

If these sensitive individuals can be identified prior to treatment and radiation therapy regimes individualized to keep the normal tissue complication rate at no more than 5%, the gains in tumour control have been estimated to be from less than 10% to as high as 40%. The higher end of the estimates has been obtained usually with the assumption that any increased radiosensitivity will be shared by both normal tissue and cancer cells (Tucker SL *et al.,* 1996; Bentzen SM, 1997; MacKay RI and Hendry JH, 1999). Indeed there have been reports where dose reductions were made to avoid radiation injury in patients with ataxia telangiectasia (see below) and tumour control has not been compromised (Abadir R and Hakami N, 1983; Hart RM *et al.,* 1987).

1.3 Can patients predisposed to develop severe radiation morbidity be identified prior to radiation treatment?

1.3a Inherited syndromes with radiation hypersensitivity

There are two well described and two less well known human syndromes which are associated with an exquisite sensitivity to ionising radiation.

Ataxia telangiectasia (AT) is an autosomal recessive condition that is associated with chromosomal fragility, immune deficiency and cancer proneness. Individuals also have ocular telangiectasias and develop ataxia. It was recognised in the 1960s that these individuals responded unexpectedly severely to ionising radiation used to treat their malignancies, some eventually perished from the radiation injury (Gotoff SP *et al.*, 1967; Morgan JL *et a.,* 1968; McFarlin DE *et al.,* 1972). Currently

wherever possible individuals with AT and cancer are treated with alternative cancer therapies.

The clinically observed hypersensitivity in AT individuals is due to a global increase in cellular radiosensitivity (Taylor AMR *et al.,* 1975) Indeed cell-lines from obligate heterozygotes have also demonstrated increased sensitivity to ionising radiation compared to normal controls (Paterson MC *et al.,* 1979) but are less sensitive than those from affected individuals. Attempts have been made to identify ATM mutations in non-syndromic patients in the 'general' cancer population who have experienced severe radiation morbidity and these have produced variable results. Five studies have found no mutations (Table 1.1). One study reported late radiation injury (including necrosis) in all 3 patients harbouring two mutations in the ATM gene, but no late injury in the 3 patients with a single mutation or the 40 with no ATM mutation (Ianuzzi CM *et al.,* 2002). A second preliminary report suggested that a disproportionate number, but not all, of prostate cancer radiotherapy patients who experience severe late effects are AT heterozygotes (Hall EJ *et al.,* 1998).

The cloning of the gene mutated in ataxia telangiectasia (*ATM*) by Savitsky et al in 1995 has paved the way for investigators to elucidate the molecular basis for the pleiotropic phenotypic features of the disease. The current knowledge of the role of the ATM protein in the area of radiation hypersensitivity will be discussed in 1.7b.

Nijmegen Breakage Syndrome (NBS) is also an autosomal recessive condition that shares many similarities with AT. Individuals also have immunodeficiency, cancer proneness and are hypersensitive to ionising radiation. However they do not have telangiestasias nor develop ataxia. Instead they are small in stature, have microcephaly and mental retardation (Taalman RDFM *et al.,* 1983).

TABLE 1.1

A summary of clinical studies examining the association of increased **radiation morbidity and ATM mutations** in cancer patients in the 'general' population.

A. Clinical studies in which ATM mutations is associated with increased radiation morbidity

Author	Number of patients	Radiation Morbidity	Type of cancer
Iannuzi CM *et al.*, 2002	46	Late subcutaneous effects, RTOG 3-4	Breast
Hall EJ *et al.*, 1998	17	Severe late sequelae, specifically proctitis or cystitis	Prostate

B. Clinical studies in which increased radiation morbidity is <u>not</u> associated with ATM mutations

Author	Number of patients	Radiation Morbidity	Type of cancer
Appleby JM *et al.*,1997	23	Mainly acute	16 breast, 7 others
Shayeghi M *et al.*,1998	41	Marked changes in breast appearance	Breast
Ramsay J *et al.*, 1998	15	Severe late	Breast
Clarke RA *et al.*, 1998	5	Severe acute	
Weissberg JB *et al.*, 1998	13	Acute and long-term	11 breast and 2 prostate
Oppitz U *et al.*, 1999	20	RTOG 3-4, acute and late	11 breast, 9 others
Izatt L *et al.*, 1999	16		Breast

Cellular features include radiosensitivity and many other similar features to those of AT. 2 groups cloned the gene responsible for this condition simultaneously in 1998 (Varon R *et al.,* 1998; Carney JP *et al.,* 1998). *Nbs1* is the human homologue of *xrs-2* and the gene product Nibrin is found to complex with hMre11 and hRad50. The function of the complex with regards to DNA damage response will be discussed in 1.7c.

In 1999, Stewart, et al ascribed the **Ataxia Telangiectasia Like Disorder (ATLD)** to a mutation in *hMre11* (Stewart GS *et al.,* 1999). ATLD has been described in only a handful of families (Hernandez D *et al.*, 1993). The affected individuals share many features of AT including progressive cerebellar degeneration, cellular radiosensitivity and chromosomal instability. The role of the hMre11/hRad50/Nbs1 complex in radiation hypersensitivity will also be discussed in section 1.7c.

Hypomorphic mutations in **DNA ligase IV** have now been described in association with extreme radiosensitivity. This was first described for a paediatric leukaemia patient who was radiation hypersensitive, developmentally normal and had no evidence of immunodeficiency (Plowman PN *et al.,*1990). The fibroblast cell line from this patient, 180BR was reported to contain a hypomorphic mutation in DNA ligase IV within a highly conserved motif encompassing the active site (Riballo E *et al.*, 1999). Subsequently, 4 paediatric patients with features of immunodeficiency and developmental growth delay were found to have mutations in *DNA ligase IV* and cell lines from these patients show pronounced radiosensitivity (O'Driscoll M *et al.,* 2001).

1.3b Radiation-sensitive individuals without any pre-treatment phenotype

Unfortunately, the vast majority of patients who develop severe radiation morbidity do not have any phenotypic characteristics that allow him or her to be identified prior to radiation treatment. It is estimated that about 5% of the population of radiotherapy patients receiving a conventional course of treatment will develop severe late morbidity (Busch D, 1994; Kagan AR, 1988). There is accumulating evidence to suggest that even for individuals with no pre-treatment phenotypes, genetic differences in normal tissue radiosensitivity contribute to the severe morbidity that is clinically observed (Busch D, 1994; Kagan AR, 1988; Geara FB *et al.,* 1998; Brock WA and Tucker SL, 2000; Andreassen CN *et al.,* 2002).

Studies have shown a positive correlation between the *in vitro* cellular or chromosomal radiosensitivity of either fibroblasts or lymphocytes and the extent of normal tissue response (Burnet NG *et al.,* 1992; Geara FB *et al.,* 1993 ; Johansen J *et al.*, 1994, Borgmann K *et al.*, 2002; West CML *et al.,* 2001; Hoeller U *et al.,* 2003) although there have been negative studies as well (Russell NS *et al.,* 1998; Peacock J *et al.,* 2000). One possible reason for this discrepancy is the 'signal' (inter-patient variability) versus 'noise' (assay variability) ratio. The two negative studies both had less favourable signal:noise ratios for reasons that are discussed in an ESTRO workshop report (Bentzen SM and Hendry JH, 1999). A signal versus noise difficulty of another, intriguing nature is well discussed in a recent paper by E Dikomey. Here the 'noise' is represented by the patients whose individual radiosensitivity falls within the normal distribution and yet develop a severe late complication of radiotherapy. Since any study of over reactors (defined as an individual whose normal tissue reaction falls outside the normal range [Burnet NG *et al.,* 1998]) is likely to have a

significant number of these patients compared to the truly sensitive individuals whose individual sensitivity falls below 1 SD from the mean ('signal'), a negative study might result (Dikomey E *et al.,* 2003).

It has also been demonstrated that first-degree relatives of breast cancer patients with increased *in vitro* radiosensitivity exhibit increased *in vitro* radiosensitivity compared to normal controls (Burrill W *et al.*, 2000). Rogers PB *et al.,* describe 3 paediatric cancer patients who were phenotypically normal at presentation and whose radiation hypersensitivity has been attributed to underlying genetic defects. 2 were attributed to the ataxia-telangiectasia gene and 1 to DNA ligase IV (Rogers PB *et al.,* 2000).

However there are as yet no tests that are in routine clinical use for the identification of individuals who may have a heightened response to radiation treatment. In spite of the studies that have positively correlated *in vitro* cellular or chromosomal radiosensitivity with the extent of normal tissue response, there is still insufficient sensitivity or specificity with these tests for them to be applied as predictive assays. Moreover assessment of cellular radiosensitivity usually involves establishing cell-lines and then either a colony forming assay or a proliferation assay to measure the survival following ionising radiation treatment. The whole procedure takes at least 4-6 weeks to perform while the patient often requires treatment to commence before such survival results are available.

Due to the need for more rapid (but reliable) tests for the identification of patients with radiation hypersensitivity, others (Kearsley JH *et al.*, 1998; Barber JBP *et al.*, 2000) have evaluated the use of methods which measure chromosomal sensitivity to radiation, namely the micronucleus test, and the G2 chromosomal

aberration test. These are performed on peripheral blood lymphocytes and thus do not require the establishment of cell-lines. While promising, Barber et al have concluded that these tests in their present form are unlikely to result in reliable 'stand-alone' predictive assays (Barber JBP *et al.*, 2000).

Yet another strategy for the development of predictive test/s is to identify genes implicated in radiation injury. This is likely to involve differences in the expression and activity of a large number of genes (Brock WA and Tucker SL, 2000; Andeassen CN *et al.,* 2002). Identification of the gene and gene products involved in normal tissue radiation response could pave the way for the development of rapid, and reliable tests perhaps using high through-put microarray technology (Peters LJ and McKay M, 2001; Russell NS and Begg AC, 2002). To this end, ESTRO has launched the GENEPI project (Genetic Pathways for the Prediction of the Effects of Irradiation) which ultimately aims to identify and quantify the impact of genetic determinants of clinical radiosensitivity and establish powerful predictive assays ("radiogenomics") for routine clinical use (Baumann M *et al.,* 2003).

1.4 Molecular Basis of Radiation Sensitivity

1.4a DNA damage induced by ionising radiation

Direct and indirect action of radiation

Ionising radiation interacts with biological material to produce its effects by two main mechanisms: direct and indirect actions. When any form of ionising radiation is absorbed in biological material, the atoms of the target, eg, DNA may be excited or ionised by the x- or gamma-rays. This is known as the direct action of radiation. However if the x- or gamma- rays first ionises water within the cell to form

free radicals, which then diffuse to and damage the critical targets, this is known as the indirect action of radiation (Hall E, 1994).

Energy deposition events

The energy deposited by ionising radiation has been categorised into three types: spurs, blobs and short tracks (Mozumder A and Magee JL, 1966). A spur is about 4 nm in diameter (about twice that of the DNA double-helix), contains less than 100 eV of energy and involves on average 3 ion pairs. A blob is about 7 nm in diameter, contains between 100 and 500 eV of energy and involves about 12 ion pairs. About 95% of events from x- or gamma-rays are spurs and 5% are blobs. Put another way, in a typical cell volume of 1.5 pl, a dose of 1 Gray will produce 75, 000 spurs and 4, 000 blobs. Short tracks involve energy amounts greater than 500 eV.

Types and yields of the various DNA lesions

The predominant cellular target responsible for the biological effects of radiation is deoxyribose nucleic acid (DNA) (Ward JF, 1986; Iliakis G, 1991; Olive PL, 1998). The cell membrane is also damaged during irradiation, initiating signalling events that involve the formation of ceramide, and activation of the stress-activated protein kinase (SAPK/JNK) cascade (Verheij M *et al.,* 1998). This signalling pathway is important in radiation-induced apoptosis (Jonathan EC *et al.,* 1999).

Within DNA, ionising radiation produces: double-strand breaks, single strand breaks, base damage, sugar damage, DNA-DNA crosslinks and DNA-protein crosslinks. The number lesions per type of damage produced per cell per Gray is as follows (Ward JF, 1988):

	Number/Gy/diploid cell
Double strand break	40
Single strand break	500 -1000
Base damage	1000-2000
Sugar damage	800 –1600
DNA-DNA crosslinks	30
DNA-protein crosslinks	150

1.4b DNA double strand breaks are associated most closely with cell lethality

Singly and locally multiply damaged sites (LMDS)

When a moiety of DNA is damaged in isolation, John Ward has called this a "singly damaged site". The relative innocence of the single-damage lesion in DNA was suggested by experiments involving hydrogen peroxide (Ward JF *et al.,* 1985; Hoffmann ME *et al.,* 1984; Bradley MO and Erickson LC, 1981). Hydrogen peroxide at 0°C was found to be proficient at in producing single-strand breaks (SSB) in the DNA of Chinese hamster fibroblasts but inefficient at producing double-strand breaks (DSB). Cell killing by hydrogen peroxide at 0°C was however inefficient. JF Ward then went on to suggest that ionising radiation, which was a lot more efficient at killing cells than hydrogen peroxide, had to produce damage in which more than one moiety of DNA in a localised region was damaged. To this region of DNA, he gave the term "locally multiply damaged sites" (LMDS). LMDS can be produced either in the same strand or in opposite strands. If sufficiently close LMDS are produced in opposite strands, then a DSB is formed and this he has hypothesised to be the lethal lesion (Ward JF, 1985). Other evidence that point to DSBs as the lethal lesion

includes the correlation of chromosome aberration with the induction of DNA DSB. Additionally incorporation of radionuclides efficient in producing DSB, eg, ^{125}IdU leads to efficient cell killing.

1.5 Models of radiosensitivity

Table 1.2 summarises some of the published data on the genetic mutations that have been found to date in human syndromes, human and mammalian cell lines and animal models (excluding transgenic mice) that are known to be hypersensitive to ionising radiation. The mutations involve predominantly two pathways: that involved in DNA damage response and repair of DNA DSBs (Table 1.2). This is not surprising given that the important biological target for ionising radiation effects is DNA and that the type of DNA damage that correlates best with lethality is the DSB. These pathways will be reviewed with respect to mammalian systems with mention of prokaryotes and other eukaryotic organisms where relevant. Where a protein/protein complex has more than one primary function, it will be discussed under the function about which most is known.

1.6 DNA Double-Strand Break Repair

The two major pathways of double strand break repair are non-homologous end joining (NHEJ) and homologous recombination (HR).

1.6a Homologous Recombination

Homologous recombination (HR) is the predominant pathway by which DNA DSBs are repaired in micro-organisms. This process makes use of the sequence

TABLE 1. 2

Genetic mutations in human syndromes, human and mammalian cell lines and animal models associated with hypersensitivity to ionising radiation.

The mutants in complementation groups XRCC1, XRCC2, XRCC3 and XRCC9 are predominantly sensitive to alkylating agents and only mildly sensitive to ionising radiation while those in groups XRCC4-8 are extremely sensitive to ionising radiation and radiomimetics (Thacker J and Zdzienicka MZ, 2003). Please refer to the text for references.

	Gene mutated in humans	Predominant Function
Human syndromes		
Ataxia Telangiectasia	*ATM*	DNA damage signalling
Nijmegen Breakage Syndrome	*NBS1*	DNA damage signalling
Ataxia Telangiectasia Like Disorder	*hMre11*	DNA damage signaling
Human cell-lines		
180 BR (Fibroblast)	*LIG4*	DNA DSB repair: NHEJ
411 BR (Fibroblast)	*LIG4*	DNA DSB repair: NHEJ
FB 2303 (Fibroblast)	*LIG4*	DNA DSB repair: NHEJ
LB 2304 (Lymphoblastoid)	*LIG4*	DNA DSB repair: NHEJ
M059J (Glioma)	*DNA-PKcs*	DNA DSB repair: NHEJ
Animal Models		
Murine, equine, canine SCID	*DNA-PKcs*	DNA DSB repair: NHEJ
Mammalian cell lines: X-Ray Cross Complementing Groups		
XRCC1	*XRCC1*	Base excision repair
XRCC2	*XRCC2*	DNA DSB repair: HR
XRCC3	*XRCC3*	DNA DSB repair: HR
XRCC4	*XRCC4*	DNA DSB repair: NHEJ
XRCC5	*Ku80*	DNA DSB repair: NHEJ
XRCC6	*Ku70*	DNA DSB repair: NHEJ
XRCC7	*DNA-PKcs*	DNA DSB repair: NHEJ
XRCC8	*Unknown*	
XRCC9	*FANCG*	DNA DSB repair: HR

identity between sister chromatids on the same chromosome or the maternal and paternal copies of the same chromosomal region for repair damage and hence is of high fidelity (Thacker J, 1999). In mammalian cells, HR occurs preferentially between sister-chromatids, with homologous chromosomes and sequence repeats on heterologous chromosomes used less frequently (Johnson RD and Jasin M, 2000). The repair of DSBs by HR is of high fidelity since the process utilizes information on a homologous chromosome or sister chromatid. In higher organisms, this process is important during the S- and G2-phases of the cell cycle (Hendrickson EA, 1997; Takata M *et al.,* 1998; Sonoda E *et al.*, 1998).

The association of radiation sensitivity with HR defects was initially made in the budding yeast, *S. cerevisiae*. In the late 1960s a number of X-ray sensitive mutants were isolated and from these studies, the *RAD52* epistasis group of mutants was identified (Game JC and Mortimer RK, 1974).

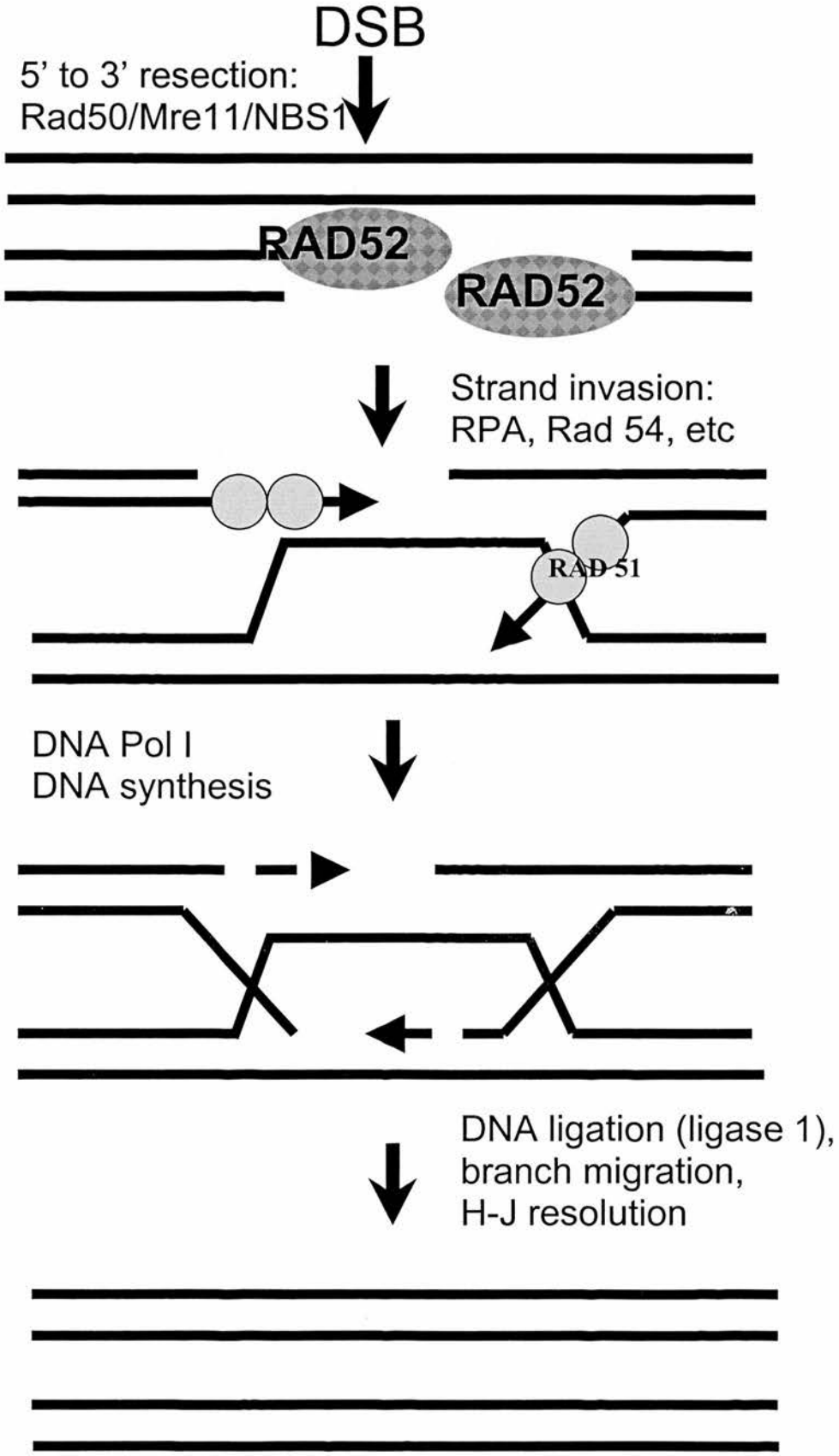
HR occurs by 3 major mechanisms: gene conversion, break induced replication and single-strand annealing (Haber JE, 2000). The classic model for the repair of DNA DSBs by HR (Resnick MA, 1976; Szostak JW *et al.,* 1983), also known as gene conversion, has the following stages (Figure 1.1):

- Initiation
- Homologous pairing and DNA strand exchange
- DNA heteroduplex extension (branch migration)
- Resolution

Figure 1.1

Model of Homologous Recombination

(Modified from Jackson SP, 2002)


DSB
5' to 3' resection:
Rad50/Mre11/NBS1
RAD52
RAD52
Strand invasion:
RPA, Rad 54, etc
RAD 51
DNA Pol I
DNA synthesis
DNA ligation (ligase 1),
branch migration,
H-J resolution

Initiation (Presynapsis)

When DNA DSBs occur, broken DNA ends are recognised, possibly by the RAD 52 protein. Following this, nucleolytic processing of the DNA ends, probably by the Rad50/Mre11/NBS1 complex, occurs to produce a single stranded 3' overhang.

Homologous pairing and DNA strand exchange

Rad51 is assembled onto the ssDNA to form a nucleoprotein filament which is also called a presynaptic filament. Rad52 and replication protein A (RPA) aid the assembly of the nucleoprotein filament in addition to ancillary eukaryotic-specific factors discussed in the following section under Rad51.

The nucleoprotein filament then searches for homologous duplex DNA and when the search is successful, invades the homoduplex DNA and forms a joint molecule through strand exchange. This stage is also known as synapsis.

DNA heteroduplex extension (Postsynapsis)

The region of DNA heteroduplex is extended by branch migrating the crossover point (also known as a Holliday Junction) and DNA synthesis which requires DNA polymerases and their accessory factors. In prokaryotes, RuvAB of the RuvABC complex promote branch migration.

Resolution

The heteroduplex is separated through resolution of the Holiday Junction (HJ) by the RuvC protein of the RuvABC complex in E. Coli and completion of DNA repair is by DNA ligase I. A complex analogous to the RuvABC complex has been isolated from mammalian cells that is capable of branch migration activity and HJ resolution (Constantinou A *et al.,* 2001).

If repeat sequences are uncovered within the single stranded 3' overhang after the initial nucleolytic processing, single-strand annealing (SSA) between the repeated sequences may occur instead gene conversion (Helleday T, 2003). The product formed by SSA contains deleted sequences and this pathway is thus error prone.

Table 1.3 (from Thompson LH and Schild D, 1999) summarises the proteins known to be involved in HR in humans. Some of these proteins will be further discussed with an emphasis on the process as it occurs in eukaryotes.

Rad51

Rad51 was first identified in S cerevisiae in 1992 as a homologue of the E coli recombination protein Rec A (Shinohara A *et al.*, 1992). Rad51 homologues from higher eukaryotes, including human Rad51 were identified in 1993 (Shinohara A *et al.*,1993). Yeast rad51 mutants are defective in DNA DSB repair and in the formation of viable spores (Friedberg E, 1995). HsRad51 shares 83% homology with SCRad51 and 55% homology with E coli Rec A. Rad51mRna has been found to be significantly increased during meiosis, and is also inducible by DNA-damaging agents in mitotic cells. In mouse and chicken, there was an interesting tissue specificity of expression: with high expression in spleen and thymus (implying a role for V(D)J recombination and in the testis and ovary cells, consistent with a major role in meiotic recombination (Bezzubova O *et al.*, 1993). No human mutant has yet been identified; the mouse knockout is embryonic lethal.

Human Rad51 has an ATP-dependent DNA binding activity and a DNA-dependent ATPase. Its role in HR repair of DNA DSBs is in the search for homologous sequences and after finding such sequences, to catalyse strand exchange.

TABLE 1.3

Key Proteins in Homologous Recombination

(from Thompson LH and Schild D, 1999)

Protein	Size (a. a)	Gene location	Rodent mutant	Protein interactions
HsRad51	339	15	Mouse KO embryonic lethal	HsRad52, HsRad54, BRCA1, BRCA2, p53, c-Abl, XRCC3
Rad51B	350	14q23-24.2	None	Rad51C
Rad51C	376	17q	None	XRCC3, Rad51B, Rad51D
Rad51D	328	17q11.2-1.2	None	XRCC2, Rad51C
XRCC2	280	7q36.1	V79 irs 1	Rad51D
XRCC3	346	14q32.3	CHO irs 1SF	HsRad51, Rad51C
HsRad52	418	12p13.3	Mouse KO viable	HsRad51
HsRad54	747	1p32	Mouse KO viable	HsRad51
HsRad50	1312	5q23-31	None	Mre11
HsMre11	708	11q21	KO is lethal in mouse ES cells	HsRad50
BRCA 1	1863	17q21	Mouse KO embryonic lethal	HsRad51, BASC (see text)
BRCA 2	3418	13q	Mouse KO embryonic lethal	HsRad51

However, unlike RecA, ATP hydrolysis and recombination reaction by Rad51 in vitro are very inefficient (Baumann P and West SC, 1998a). Rad51 activities are enhanced by interactions with ssDNA binding protein, RPA, Rad52 and Rad55-57 in S. cerevisiae (Shinohara A and Ogawa T, 1999).

In S. cerevisiae, Rad 52 mutations have the most pronounced recombination deficient phenotype (Paques F and Haber JE, 1999). Surprisingly in vertebrate cells, Rad52p plays a less central role in that Rad52 deficient mice are viable and not sensitive to ionising radiation (Yamaguchi-Iwai *et al.*, 1998; Rijkers T *et al.,* 1998.)

Ancillary eukaryotic-specific factors either facilitate the activity of the primary strand exchange proteins or promote strand transfer themselves. These factors include Rad54, Rad59, Rad51B Rad51C, Rad51D, Rad55, Rad 57, Xrcc2 and Xrcc3 (Cromie GA *et al.,* 2001). S. cerevisiae Rad54 is required for D loop and heteroduplex DNA formation. Rad 54 deficient mice are also viable and sensitive to ionizing radiation at the embryonic but not at the adult stage (Essers J *et al.,*1997; Essers J *et al.*, 2000). Similarly a rad54 deficient chicken cell line is also sensitive to ionizing radiation (Bezzubova O *et al.,* 1997).

5 Rad51 paralogs have been described in mammalian cells, Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3, that share less than 50% sequence homology with Rad51 (Sonoda E *et al.,* 2001). *XRCC2* (Tanbini CE *et al.,* 1997) and *XRCC3* (Tebbs RS *et al.,* 1995) were identified in rodent cell lines, irs1 (Jones NJ *et al.,* 1987) and irs1SF (Zdzienicka MZ and Simons JW, 1987) respectively. Their hallmark is extreme sensitivity to DNA cross-linking agents such as mitomycin C and they are also moderately radiosensitive. Irs1 was found to have 100-fold reduction in HR induced by double-strand breaks compared to its parental cell line leading to the

conclusion that *XRCC2* is involved in HR repair (Johnson RD *et al.*, 1999). The disruption of *XRCC2* leads to embryonic lethality in mice (Deans B *et al.*, 2000). Similarly, in irs1SF, in which XRCC3 is deficient, error-free homology-directed repair of DNA DSBs is reduced 25-fold and restored to wild-type levels through XRCC3 expression (Pierce AJ *et al.*,1999; Brenneman MA *et al.*, 2000).

BRCA2

BRCA2 is a tumour suppressor gene mutated in a proportion of breast cancer patients with a familial incidence. The 3 418 amino acid human protein has no significant homology to any known protein. *Brca2*$^{-/-}$ mouse stem cells are radiation hypersensitive (Sharan SK *et al.*, 1997). The role of BRCA2 in homologous recombination was initially suspected due to its association with MmRad51 (Sharan SK *et al.*, 1997). More recently, evidence has ruled out a role for BRCA2 in non-homologous end joining (Patel KJ *et al.*, 1998).

Hypomorphic *BRCA2* mice mutants have been generated and these have impairment of HR (Moynahan ME *et al.*, 2001; Tutt 2001). In the former study, Rad51 dependent homology directed repair of site-specific double-strand breaks which are introduced into a chromosomal substrate by the I-SceI endonuclease, was impaired. CAPAN-1, a human pancreatic adenocarcinoma cell line which carries a single *BRCA2* allele is also severely compromised in HR (Xia F *et al.*, 2001; Moynahan ME *et al.*, 2001b) in which there is also impairment of DNA damage-induced RAD51 foci formation (Yuan 1999).

BRCA 2 interacts with Rad51 through 6 of its 8 evolutionarily conserved BRC repeat motifs (Wong AKC *et al.*, 1997; Chen PL *et al.*,1998) and controls Rad51

function in 2 ways. The first is in the nuclear localisation of Rad51 which is inefficiently transported into the nucleus in certain BRCA2 deficient cells (Davies AA *et al.*, 2001). Secondly, BRCA2 interacting with RPA at the DSBs helps load RAD 51 onto the DNA or to organize the nucleoprotein filament (Yang H *et al.,* 2002).

BRCA1

BRCA1 mutations are found in a significant percentage of familial breast and especially breast and ovarian cancer kindreds. BRCA1 mutant mouse cells are hypersensitive to gamma-irradiation (Shen SX *et al.*, 1998). Brca1 deficient mouse embryonic cells have impaired repair of chromosomal double-strand breaks by homologous recombination and this is accompanied by elevated rates of non-homologous end joining (Moynahan ME *et al*, 1999).

BRCA1 is an 1863 residue protein whose role in homologous recombination was first observed due to its association with Rad51 in mitotic and meiotic cells using pulldown assays with cell-free extracts (Scully R *et al.*, 1997). It is now thought that the observed interaction between Rad51 and BRCA1 might have been mediated through BRCA2 (West SC, 2003) which forms complexes with BRCA1 in vivo (Chen J *et al.*, 1998). BRCA1 was later found to co-localise with RAD 50, Mre11 and NBS1 upon irradiation (Zhong Q *et al.,* 1999). This is preceded by the phosphorylation of histone H2AX (Paull T *et al.,* 2000) and there is impaired recruitment of BRCA1 to these repair foci in H2AX -/- mice (Celeste A *et al,* 2002) and embryonic stem cells (Bassing CH *et al.,* 2002). Paull *et al* also found that BRCA1 either co-localised with RAD 50 or with RAD51 but not both within the same cell.

Apart form its role in homologous recombination, BRCA1 is also part of a large complex of proteins known as BASC (BRCA1-associated genome surveillance complex) that contains ATM, Nbs1-MRE11-RAD50, mismatch proteins MSH2/6 and MLH2 and Bloom's helicase (see 1.8) (Wang Y *et al.,* 2000). Damage associated phosphorylation of BRCA1 by multiple kinases precedes formation of repair complexes; these kinases include ATM (Cortez D *et al.,*1999), Chk2 (Lee JS *et al.,* 2000) and ATR (Tibbetts RS *et al.,* 2000) (See also 1.7b).

1.6b Non-Homologous End Joining

During non-homologous end joining (NHEJ) of two DNA ends, no sequence homology is required between the two recombining ends. In this process the two ends are re-ligated together, sometimes after limited processing at the termini. As such, NHEJ is an error prone process with small sequences usually deleted (Khanna KK and Jackson SP, 2001).

The classical theory had been that, in contrast to yeast, mammalian cells repaired DNA double-strand breaks predominantly by NHEJ (Roth DB and Wilson JH, 1985). However, it is now established that homologous recombination also has a crucial role in mammalian cells (Liang F *et al.,* 1998). The predominant process appears to be dependent on cell type, stage of development or phase of the cell cycle. In G1/G0 phase, the great majority of DSBs are rejoined by NHEJ (Lee SE *et al.,* 1997; Takata M *et al.,* 1998).

In mammalian cells, the process of NHEJ involves the DNA-PK heterotrimer of proteins, XRCC4/ DNA ligase IV and Artemis (Figure 1.2). The former is made up of a large catalytic sub-unit (DNA-PKcs) and the DNA end-binding protein Ku

Figure 1.2

Model of Non-Homologous End Joining

(Modified from Jackson SP, 2002)

KU70
KU80
DNA-PKcs
DNA-PKcs
KU70
KU80
MRE11
NBS1
RAD50
ARTEMIS
XRCC4
DNA
ligIV

70/80. When a DNA double-strand break arises as a result of ionising radiation, it is first bound by Ku. After binding, Ku recruits DNA-PKcs and activates its kinase activity. Prior to ligation, the two DNA ends are held together in physical proximity in a process called synapsis (DeFazio LG *et al.* 2002). During synapsis, the DNA ends are processed and Artemis, the most recent component of NHEJ elucidated, is a candidate nuclease for this (Moshous D *et al.,* 2001). XRCC4 is then recruited and the repair is completed by ligation of the ends, a role ascribed to DNA ligase IV (Karran P, 2000).

DNA-PK is one of two kinases known to be specifically activated by DNA termini, the other being ATM (Hartley KO *et al.,* 1995). It is a serine/threonine kinase that is made up of a large catalytic subunit, termed DNA-PKcs (also known as PRKDC) and a regulatory factor termed Ku.

DNA-PKcs is about 460 kDa encoded by a 14 kb mRNA (Hartley KO *et al.,* 1995). The only region with strong homology to other proteins is the C-terminal 380 amino acid residues which is highly related to the catalytic domains of the PI 3-kinase family that also include ATM, ATR and FRAP. The murine *scid* mutation is a nonsense mutation that results in premature termination of translation within its kinase domain (Danska JS *et al.,* 1996).

The molecular structure of the catalytic subunit, DNA-PKcs suggests it has a role as a molecular scaffold whereby short stretches of single-stranded DNA may be aligned prior to religation. It has a potential DNA-binding groove and an enclosed cavity with three apertures through which single-stranded DNA could pass (Leuther KK *et al.,* 1999). The activation of DNA-PKcs is triggered by its interaction with a single-stranded DNA region derived from a DSB (Hammarsten O *et al.,* 2000;

Martensson S and Hammarsten O, 2002). No *in vivo* substrates for DNA-PKcs has been identified though likely candidates are XRCC4 (Leber R *et al.,* 1998) and replication factor A2 (Wang H *et al.*, 2001a). What has been demonstrated is that autophosphorylation of DNA-PKcs occurs *in vivo* in response to ionizing radiation and is required for the repairs of DSBs by NHEJ (Chan DW, *et al.,* 2002).

Information about the role played by DNA-PK in NHEJ and radiation sensitivity has come from three main sources: mutant rodent cell lines, spontaneous mammalian (rodent, equine and canine) mutants and transgenic mice and a single mutant human tumour cell line.

DNA-PKcs is the product of the murine *scid* gene (Blunt T *et al.*, 1995; Kirchgessner CU *et al.,* 1995). The SCID (severe combined immuno-deficiency) mouse was identified in 1983 due to the virtual absence of mature T and B lymphocytes from a defect in VDJ recombination (Bosma GC *et al.,* 1983). Later it was also found to be profoundly sensitive to ionising radiation and to be defective in DNA double-strand break repair (Fulop GM and Phillips RA, 1990; Biedermann KA *et al.,*1991). The gene was also found to map to the same region as the *XRCC7* gene (Sipley JD, *et al.,* 1995). The *XRCC7* (X-ray cross-complementing) gene complements cells which were assigned to the IR7 group of radiosensitive mutant rodent cell lines (Zdzienicka MZ, 1995). Reduced DNA-PKcs expression and DNA-PK activity is also the underlying mechanism in SCID in Arabian foals (Wiler R *et al.,* 1995). The condition was described initially in 1973 in animals which were profoundly deficient in B and T lymphocytes and where cells derived from them are hypersensitive to ionising radiation. mRNA levels are not diminished suggesting that the protein is unstable in equine SCID The latest animal model of spontaneous SCID

is that of Jack Russell Terriers. Severe immunodefieciency has again been shown to be due to defective V(D)J recombination and DNA-PKcs deficiency. It is also interesting that the severity of the phenotype is worst in horses, with canine scid intermediate and murine scid the least severe and the degree of severity is inversely proportional to the relative DNA-PK activity in expressed in normal fibroblasts derived from these three species (Meek K *et al.*, 2001).

Further understanding of the role of DNA-PKcs has come from characterising several independent X-ray sensitive cell-lines which all belong to the XRCC7 group and include V-3, irs-20, XR-C1, XR-C2, equine *scid*, SX-9 and M059J (Zdzienicka MZ, 1999). M059J is unique in the list, being the only human cell line with a DNA-PK deficiency. Derived from a human glioma, M059J has no detectable protein expression (Lees-Miller SP *et al*, 1995) and low ATM expression (Chan DW *et al.*, 1998). However the radiation sensitive phenotype was complemented by a fragment of human chromosome 8 that contains a copy of DNA-PKcs thus showing that the defect causing radiosensitivity in M059J is in the *DNA-PKcs* gene. (Hoppe BS *et al.*, 2000). More recently, a single nucleotide deletion in exon 32 was identified in the DNA-PKcs gene which would give rise to a frameshift mutation and could account for the biological properties of these cells (Anderson CW *et al.*, 2001).

The kinase activity of DNA-PKcs is crucial for the function of the protein in regards to DNA DSB rejoining (Kurimasa A *et* al., 1999) and in the SCID mouse, the C-terminal domain required for kinase activity is missing (Beamish HJ *et al.*, 2000).

Substrates phosphorylated *in-vitro* by DNA-PKcs include XRCC4 (see below), WRN (the Werner syndrome protein) and Artemis. Physiological targets of the protein continue to remain elusive although auto-phosphorylation of DNA-PKcs

has been found at Thr 2609 in vivo in a Ku-dependent manner in response to ionizing radiation (Chan DW *et al.*, 2002). In okadaic acid-treated human cells, DNA-PKcs is also phosphorylated in vivo at Thr2609, Ser2612, Thr2638 and Thr2647 (Douglas P *et al.,* 2002). Auto-phosphorylation inactivates the serine/threonine kinase activity of DNA-PK resulting in dissociation of DNA-PKcs from the Ku-DNA complex and is thought to be an important regulatory mechanism for the enzyme complex (Chan D and Lees-Miller SP, 1996).

Knockout mice have been generated that are null for DNA-PKcs (Jhappan C *et al.,*1997; Gao Y *et al.*, 1998a) or ablated for its kinase domain (Taccioloi GE *et al.,*1998). The knockout mice are normal in size with no gross phenotypic abnormalities except radiosensitivity and impaired lymphocyte development.

To date, DNA-PKcs has only been identified in vertebrates, in contrast to Ku which has been found in all eukaryotes examined.

Ku was first identified from the sera of patients with autoimmune diseases (Mimori T *et al.,* 1981). It is a highly abundant nonspecific DNA-binding protein comprising two subunits of approximately 70 and 83 kD, referred to as Ku70 and Ku80 or Ku86 respectively. (Mimori T and Hardin JA, 1986). The cloning of the gene encoding Ku80 revealed that it was also the product of the *XRCC5* gene which complements radiosensitive rodent cell-lines belonging to the IR5 group (Taccioli GE *et al.*, 1994). The gene is located on chromosome 2q33-35.

The XRCC5 mutants include *xrs* series, the XR-VB and the *sxi* series. Ku mutants lack DNA end-binding and DNA-PK kinase activities with resulting defects in DSB repair and V(D)J recombination. Lack of the Ku80 protein is accompanied by absence of Ku 70 protein and DNA-PK activity but DNA-PKcs levels are unaffected

(Zdzienieka MZ, 1999). No rodent mutant defective in Ku70 has yet been identified nor is there any human cell-line with spontaneous mutations in either subunit.

Knockout mice for both Ku70 and Ku80 have been generated. Ku70 and Ku80 knockout mice are very similar in phenotype (Nussenzweig A *et al.,* 1996; Nussenzweig A *et al.,*1997; Gu Y *et al.*, 1997; Ouyang H *et al.,*1997). Both $Ku70^{-/-}$ and $Ku80^{-/-}$ mice are small in size, weighing 40-60% of the weight of control littermates. They are hypersensitive to ionising radiation and severely T and B lymphocyte deficient. In the case of $Ku70^{-/-}$ (but not $Ku80^{-/-}$) mice, the majority of animals develop T-cell lymphoma (Li GC *et al.,*1998).

One of the earliest insights into the role of Ku in NHEJ was provided by the discovery that DNA-PK binding to DNA ends was required for kinase activation and that this binding was presumably by means of the Ku subunits (Gottlieb TM and Jackson SP, 1993). Indeed Ku-DNA complexes visualised by scanning force microscopy is consistent with two DNA molecules held together by Ku (Yaneva M *et al.,* 1997). The crystal structure of human Ku heterodimer has been determined to be a dyad-symmetrical molecule with a preformed ring that encircles duplex DNA (Walker JR *et al.*, 2001). Once bound, Ku is postulated to do at least two things: it protects the DNA ends from degradation by exonuclease and recruits DNA-PKCS to the site of break (Chu G, 1997). The carboxy terminal portion of Ku80 has been found to be important for DNA-PKcs activation – the deletion of this region of Chinese hamster Ku80 imparts a phenotype similar to DNA-PK deficiency (Singleton BK *et al.*,1999). Ku also facilitates ligation of DNA ends by aligning the ends (Ramsden DA and Gellert M, 1998), recruits the DNA ligase IV-XRCC4 complex to the break and stimulates ligation by ligase IV (McElhinny SAN *et al.,* 2000).

The latter complex of XRCC4 and DNA ligase IV complete the process of NHEJ by literally seeing to the religation of the DNA ends (Karran P, 2000). *Xrcc4* was cloned by functional complementation of the XR-1, a radiosensitive CHO cell line (Li Z *et al.*,1995). The gene product is a 334 amino acid nuclear protein. XRCC4 forms an extremely tight complex with DNA ligase IV in mammalian cells (Critchlow SE *et al.*,1997; Grawunder U *et al.*,1997). Human DNA ligase IV is a 911 amino acid protein whose activity *in vitro* is stimulated by its association with XRCC4. At the same time that the association was described, the role of DNA ligase IV as providing the end-joining activity was supported by the finding that yeast DNA ligase IV mediated NHEJ (Wilson TE *et al.*, 1997).

Further evidence for the participation of the XRCC4-DNA ligase IV complex to participate in the same pathway have come from several observations. DNA-PK has been shown to phosphorylate XRCC4 *in vitro* and Ku has been demonstrated to interact with XRCC4-DNA ligase IV at DNA ends (Chen L *et al.*, 2000). Exposure of to ionising radiation resulted in phosphorylation of XRCC4 as evidenced by an electrophorectic mobility shift; M059J cells which lack DNA-PKcs did not demonstrate a similar shift (MatsumotoY *et al.*, 2000). However the physiological significance of XRCC4 phosphorylation by DNA-PK is unknown. XRCC4, with mutations of 2 major DNA-PK sites (ser 260 and ser 318) to alanine, was still able to complement the radiosensitivity and V(D)J recombination deficiencies in an XRCC4 negative cell line (XR-1) (Yu Y *et al.*, 2003).

DNA ligase IV and *Artemis* are the only NHEJ genes in which mutations have been found in human disease. A hypomorphic mutation in DNA ligase IV was reported within a highly conserved motif encompassing the active site in a leukaemia

patient who was radiation hypersensitive (Riballo E *et al.*, 1999). It is significant that the individual was developmentally normal and had no evidence of immunodeficiency (Plowman PN *et al.*,1990). Using a fibroblast cell-line established from this individual (180BR), genetic evidence for the involvement of DNA ligase IV in the DNA-PK dependent pathway of NHEJ in mammalian cells was demonstrated (Wang H *et al.*, 2001b). Subsequently, 4 patients with features of immunodeficiency and developmental growth delay were found to have mutations in *DNA ligase IV* and cell lines from these patients show pronounced radiosensitivity (Table 1.2) (O'Driscoll M *et a.*, 2001).

Mice deficient for XRCC4 or DNA ligase IV are not viable and die during late embryonic development as a consequence of neuronal defects (Barnes DE *et al.*, 1998; Frank KM *et al.*,1998; Gao Y *et al.*, 1998).

A subset of children with severe combined immunodeficiency (SCID) have an associated increase in cellular radiosensitivity and the condition is denoted RS-SCID. These children have been found to have loss-of-function mutations in *Artemis*, which is located at chromosome 10p (Moshous D *et al.*, 2001). More recently hypomorphic mutations associated with low but detectable number of B and T lymphocytes, have been described in four patients (Moshous D *et al.*, 2003). The gene codes for a 77 kDa protein that belongs to the metallo-β-lactamase superfamily. Artemis has 3', 5' exonuclease activity and together with DNA-PK cleaves 3' and 5' overhangs, as well as opening the hairpin intermediates during V(D)J recombination (Ma Y *et al.*, 2002). Artemis, like DNA-PKcs has to date been found only in higher species. Artemis-deficient mice are remarkably similar to DNA-PKcs deficient mice (Rooney S *et al.*, 2002). The mice are normal in size, have increase in cellular radiosensitivity and scid.

NHEJ likely involves more proteins; requirement for an additional, as yet uncharacterised factor, has been identified in a patient with SCID (Dai Y *et al.,* 2003).

1.7 DNA damage response

The processes of homologous recombination and non-homologous end-joining are called into play when cells experience DNA damaging agents such as ionising radiation. The mechanism by which cells sense the damage and the signalling processes that eventually lead to the assembly of the proteins involved in the repair processes are constantly being elucidated in yeast and in higher organisms. Genes that are known to be mutated with a radiosensitive phenotype and involved in the DNA damage response include *S cerevisiae RAD 9*, and human *ATM*, *NBS/MRE 11/ RAD 50* complex.

1.7a *S cerevisiae RAD9* Gene as a paradigm

It has long been observed that cells treated with DNA-damaging agents, in particular ionising radiation, arrest cell cycle progression in the G_2 phase of the cell cycle (Walters RA *et al.,*1974; Tobey RA *et al.,*1975). The RAD 9 mutant of *Saccharomyces cerevisiae* is a radiation sensitive mutant that is also defective in G_2 arrest after treatment with gamma rays. Treatment of the cells with an agent that inhibits normal assembly of the mitotic spindle and thereby artificially imposing a mitotic delay restores the sensitivity of the *rad 9* mutants to wild-type levels (Weinert TA and Hartwell LH, 1988). The authors argue that efficient DNA repair can therefore occur in *rad 9* mutants and the *RAD9*-dependent response causes arrest of cells in G_2 until DNA damage is repaired.

The *RAD9* gene encodes a protein with a predicted molecular mass of 148 kDA. The only phenotype conferred by deletion of the gene in cells unchallenged by

DNA-damaging agents is an increased rate of spontaneous chromosome loss (Weinert TA and Hartwell LH, 1990). Rad 9 is phosphorylated in response to DNA damage and that phosphorylated Rad 9 has a role in modulating protein-protein interactions and activating downstream kinases, for example RAD53 (Sun Z *et al.,* (1998). Human Rad 9 is a target of ATM (Chen MJ *et al.,*2001).

1.7b ATM (Ataxia-Telangiectasia Mutated)

The gene mutated in ataxia telangiectasia, *ATM,* was cloned in 1995 after a 20 year search (Savitsky K *et al.*, 1995). It is localised on chromosome 11q22-23. It codes for a predominantly nuclear, 370 kDA protein kinase that bears similarity in its carboxy-terminal sequence to the catalytic domain of phosphatidylinositol-3-OH kinase (PI(3)K). Current understanding places ATM at the centre of a signalling network activated in response to DNA DSBs in mammalian cells (Shiloh Y, 2003) (Fig 1.3). The yeast homologues of *ATM* include *MEC1* in *S. cerevisiae* and *Rad3* in *S. pombe.*

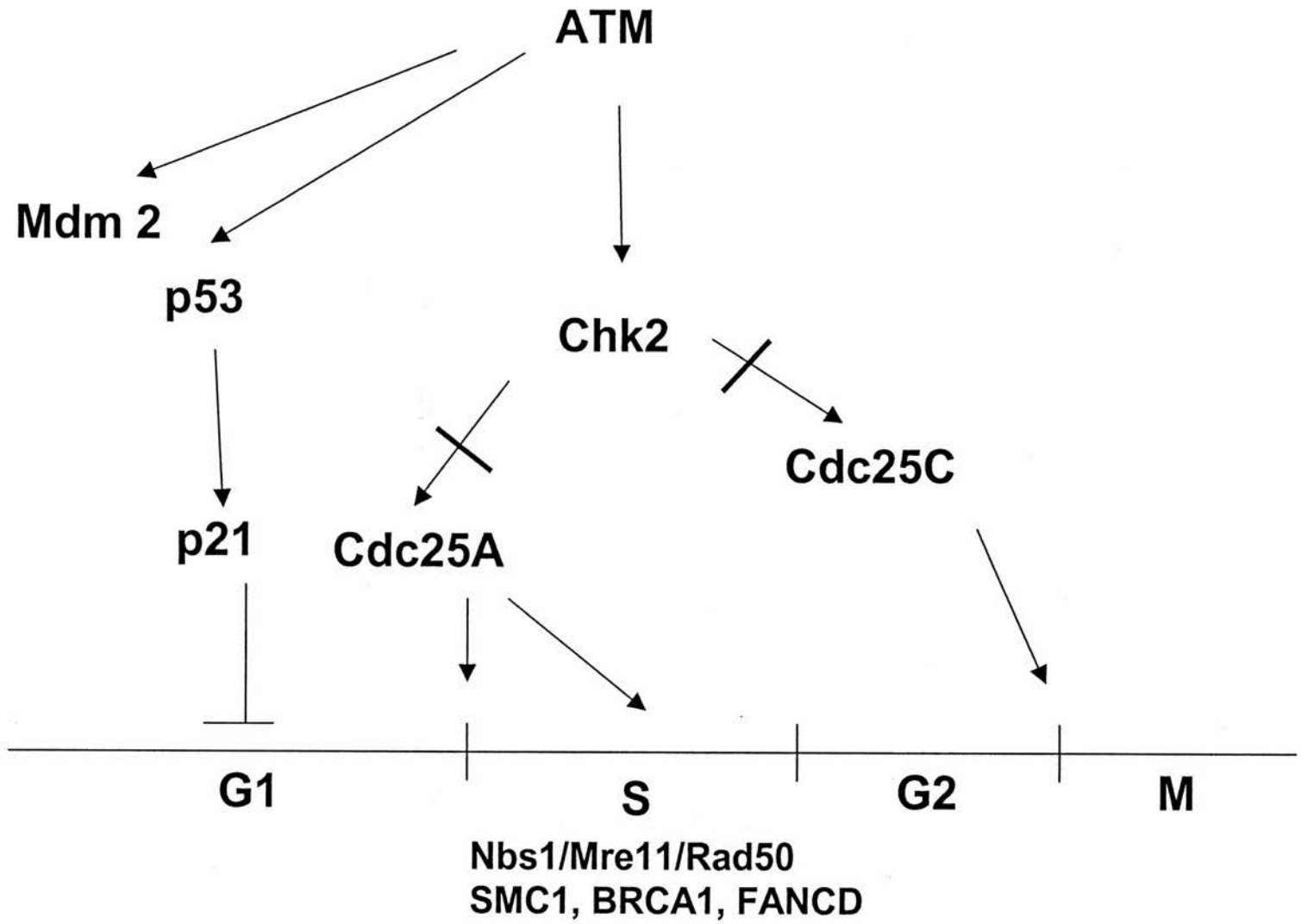
The main phenotypic features of AT cells are:

- chromosomal instability (Kojis *et al.,* 1989),
- cell-cycle checkpoint defects in G_1/S (Nagasawa H *et al.,* 1985) and G_2/M (Beamish H and Lavin MF, 1994),
- as well as S-phase, the latter also known as radioresistant DNA synthesis, ie, an inability to arrest DNA synthesis following irradiation (Houldsworth J and Lavin M, 1980; Painter RB and Young BR, 1980) and
- increased sensitivity to ionising radiation (Taylor AMR *et al,* 1975).

Figure 1.3

Central Role of ATM in the DNA damage response

(Modified from Iliakis G *et al.*, 2002 and Shiloh Y, 2003)


ATM
Mdm 2
p53
Chk2
Cdc25C
p21
Cdc25A
G1
S
G2
M
Nbs1/Mre11/Rad50
SMC1, BRCA1, FANCD

Normal cells, on exposure to ionising radiation, respond by having increased cellular levels of p53. P53 goes on to activate p21 production and consequently there is a halt in the progression from G_1 to S phase. It had been observed that AT cells showed poor induction of p53 after ionising radiation (Kastan MB *et al.*,1992). The first complete description of an ATM target provided evidence that ATM kinase phosphorylates serine 15 and 20 of p53 in response to ionising radiation (Siliciano JD *et al.,* 1997). The downstream effects will be discussed in the section below.

Exposure of cells to ionizing radiation does not change the subcellular distribution or the total amount of cellular ATM protein (Watters D *et al.*,1997; Brown K. D *et al.,* 1997). However, pre-existing ATM is rapidly activated by ionizing radiation and radiomimetic agents (Banin S *et al.,* 1998; Canman CE *et al.,* 1998; Khanna KK *et al.,* 1998). While the upstream events of this activation are not completely understood, atomic force micrographs provide compelling evidence that ATM binds directly to free DNA ends (Smith GC *et al.,* 1999). More recently, evidence has been provided that DNA DSBs trigger widespread activation of ATM through changes in chromatin structure (Bakkenist CJ and Kastan MB, 2003). Even a few DNA DSBs was sufficient to activate the majority of intracellular ATM, through the ionizing radiation induced phosphorylation of the protein at Ser-1981. ATM homodimeric or oligomeric complexes, which maintain the protein in an inactive state in undamaged cells, then dissociate and activated ATM monomers are released. However the signal that links the structurally altered chromatin with Ser-1981 phosphorylation is still undefined.

Chk2 is the human homologue of yeast *Rad53* and *Cds1*, that are required for responses to DNA damage and replication blocks in these organisms. It is a

serine/threonine kinase which is phosphorylated by ATM at Thr68 *in vivo* and *in vitro*, and thereby activated, in response to ionizing radiation (Matsuoka S *et al.*,2000; Melchionna R *et al.*, 2000). The activation of Chk2 kinase leads to the downstream phosphorylation of Cdc25C at Ser216 (Matsuoka S *et al.*, 1998; Brown AL *et al.*, 1999; Chaturvedi P *et al.*, 1999). In its phosphorylated state, Cdc25C maintains cells in G2 arrest. Chk2 also phosphorylates CDC25A (Falck J *et al.*, 2001) which marks it for destruction, thus 'locking' CDK2-cyclin E kinase in an inactive state, so contributing to the G1-S, intra-S and G2-M DNA damage checkpoints.

The intra-S checkpoint was the first to be recognized as deficient in AT cells (Painter RB and Young BR, 1980) is now seen as one of the most crucial cellular responses to DNA DSBs (Shiloh Y, 2003). The proteins involved in this checkpoint that are phosphorylated by ATM include NBS1 (see below), SMC1 (structural maintenance of chromosomes 1) (Kim ST *et al.*, 2002; Yazdi PT *et al.*, 2002) and FANCD2 (Fanconi Anaemia Complementation Group D) (Taniguchi T *et al.*, 2002) in addition to Chk2 already discussed above. This explains why abrogation of ATM function results in an impaired S-phase DNA damage response.

Whether it is the checkpoint deficiencies or a repair defect per se that accounts directly for the cellular hypersensitivity to ionising radiation of AT cells continues to be controversial. Jeggo *et al.* argue that there is evidence (Jeggo PA *et al.*,1998), that the hypersensitivity of AT cells to ionising radiation is not due to their checkpoint defects but rather to a distinct DNA repair defect. One of the lines of evidence argued by the authors is that while p53 defective cell lines also display cell cycle checkpoint defects, they are also radioresistant instead of radiosensitive (see below). Also

Lehmann *et al.* describe an AT derived cell line with normal radiation sensitivity but also displays radioresistant DNA synthesis (Lehmann AR *et al.,* 1986).

A closely related related protein is ATR (ATM-Rad3-related). This is also a PI3- kinase like protein whose homologues in yeast, Mec1 and Rad3 have roles in the DNA damage response pathways. ATR plays a role in the later (as opposed to the rapid, ATN dependent) response to DSBs (Tibbetts RS *et al.,* 1999) but its main role is in the response pathway to UV radiation induced damage, stalled replication forks and hypoxia (Heffernan TP *et al.,*2002; Hammond EM *et al.*, 2002). ATR mutations have thus far not been described in human diseases.

DOWNSTREAM OF ATM: P53 AND RETINOBLASTOMA PROTEIN

P53 is a tumour suppressor gene that is mutated in about 50% of human cancers and has been described as the guardian of the genome. Germline mutations of *p53* in humans result in the Li-Fraumeni syndrome in which affected individuals are prone to early onset cancer (sarcoma, breast cancer, brain tumours, leukaemia and adrenocortical carcinomas). P53 null mice, although developmentally normal, are also predisposed to tumourigenesis (Donehower LA *et al.,* 1992).

Wild type p53 has a pivotal role in many cellular processes including regulation of DNA damage-induced cell-cycle checkpoints (Kastan MB *et al.,* 1991), apoptosis (Clarke AR *et al.,* 1991) and more recently aging (Tyner SD *et al.,* 2002). The p53 protein is also thought to be important in maintaining genetic stability and inhibiting blood-vessel formation in tumours by mechanisms that are as yet unclear (Vogelstein B *et al.*, 2000).

The link between abnormal p53 function and radiation sensitivity comes from the finding that cells form the radiosenstitive syndrome, Ataxia Telangiectasia, show delayed induction of p53 levels in response to ionising radiation (Kastan MB *et al.,* 1992). Upon exposure to ionising radiation in normal cells, ATM phosphorylates p53 preferentially at Ser 15, leading directly to stabilisation of the protein and up-regulation of its levels (Siliciano JD *et al.,* 1997; Banin S *et al.,* 1998; Canman CE *et al.,* 1998; Khanna KK *et al.,* 1998). In addition, Chk2 kinase is activated and in turn stabilises p53 in response to DNA damage by phosphorylating ser20 (Hirao A *et al.,* 2000). Furthermore, MDM2 is phosphorylate by ATM on Ser 395 and this interferes with the ubiquitin-mediated degradation of p53 leading to its accumulation (Khosravi R *et al.,* 1999; Maya R *et al.*, 2001). These proteins are still phosphorylated in gamma-irradiated AT cells, but with delayed kinetics indicating alternative ATM independent pathways p53 induction by ionising radiation.

The accumulation of p53 allows it to act on its transcriptional targets. In the case of G1 arrest, this is chiefly the *p21* gene which encodes a cyclin dependent kinase inhibitor (el-Deiry WS *et al.,* 1994). Up-regulation of p21 expression inhibits cyclin D- and cyclin E-associated kinases (Dulic V *et al.*, 1994). This allows for the accumulation of retinoblastoma protein (pRb) in its hypo-phosphorylated state resulting in G1/S arrest. MDM2 is also transcriptionally activated by p53, the former then binds to the latter, stimulating ubiquitination and degradation, resulting in a negative feedback loop (Vogelstein B *et al.,* 2000).

A significant G2 arrest function has been reported for p53 (Agarwal ML *et al.*, 1995). This is partly mediated through the transcriptional activation of 14-3-

3sigmawhich sequesters cyclinB1-CDK1 complexes outside the nucleus and helps maintain the G2 block (Chan TA *et al.,* 1999; Laronga C *et al.,* 2000).

In contrast to the relationship between impaired p53 induction and radiosensitivity in AT cells, cells from transgenic mice with mutations in p53 show increased survival following ionising radiation. (Lee JM and Bernstein A, 1993). Similarly, Li-Fraumeni syndrome fibroblasts with p53 mutations demonstrated increased resistance to low dose rate ionising radiation (Sproston ARM *et al.,*1996).

1.7c NBS1/MRE11/RAD50 Complex

The Nijmegen Breakage Syndrome (NBS) was described first in 1981 (Weemaes CM *et al.,* 1981); it is a rare autosomal condition that afflicts about 70 families of mainly Eastern European descent. Although the clinical features are distinct from AT, the cellular phenotype of cells derived from individuals with NBS are virtually identical to AT cells. In 1998 2 laboratories using different methodologies independently identified the gene product that is mutated in the syndrome: Nibrin or NBS1 (Carney JP *et al.*, 1998; Varon R *et al.,* 1998). *NBS1* was localized to 8q21where it spans 50kb of genomic DNA and codes for a protein with a calculated molecular weight of 85 kDa.

In 1999, a further piece in the puzzle was filled in when the syndrome Ataxia Telangiectasia-Like Disorder (ATLD) was found to be caused by a mutation in hMre11. (Stewart GS *et al.,* 1999). Like AT and NBS cells, ATLD cells also display increased radiation sensitivity, chromosomal instability and radioresistant DNA synthesis. *hMre11* is located at 11q21. It codes for a multi-functional protein of about 80 kDa with DNA nuclease, strand association and strand-annealing activities.

The third member of the complex is Rad50. This is a 150 kDa protein that contains nucleotide binding motifs at its amino- and carboxyl-terminal ends.

In normal cells, Nibrin is constitutively associated with hMre11 and hRad50. The Mre-11/Rad50/Nibrin (M-R-N) complex is rather abundant and for the most part uniformly distributed in the nuclei of the cells. When these are exposed to ionising radiation, the proteins become associated in large foci, strictly dependent on the prior induction of double strand breaks (Nelms BE *et al.,* 1998; Petrini JH *et al.,* 1999). These foci are now known as ionising radiation-induced foci (IRIF), being one of three distinct foci formed by the Mre11 complex (Mirzoeva OK and Petrini JH, 2001). Importantly the foci are detectable at the earliest time points examined by the investigators and dissipate as the double-strand breaks are repaired. In NBS cells, the Mre11 and Rad50 are present at normal levels but are cytoplasmic in distribution and the IRIF formation are completely abolished.

Human NBS1 has weak (29%) homology to yeast Xrs2 protein. The N-terminus consists of a fork head associated (FHA) domain and a BRCA1 C-terminus (BRCT) domain. Kobayashi *et al* have shown that the FHA/BRCT domain directly binds to histone γ-H2AX and recruits the M-R-N complex to the vicinity of sites of DSBs (Kobayasihi K *et al.,* 2002). H2AX is a member of the histone protein H2a family. It is phosphorylated within seconds of exposure to ionising radiation, at Ser 139 in the carboxy- terminal portion (Rogakou EP *et al.,* 1998). This response was directly proportional to the amount of DSBs induced. ATM phosphorylates H2AX in-vitro and this phosphorylation is severely compromised in *atm -/-* cells (Burma S *et al.,* 2001). Histone γ-H2AX foci are followed by co-localisation with BRCA1 and then by either Rad50 or Rad 51 depending on the cell type (Paull T *et al.,* 2000) and

one of its postulated roles is the recruitment of repair factors to sites of DNA DSB damage (Kobayashi K *et al.,* 2002).

AT, ATLD and NBS cells also display radioresistant DNA synthesis due to a defect in the S-phase checkpoint following ionising radiation. Data by a number of independent laboratories show that ATM phosphorylates Nibrin at two or more serine residues (Ser 343 was common to all laboratories) in response to ionising radiation. This event influences S-phase checkpoint. Cells expressing mutant proteins that cannot be phosphorylated by ATM at Ser343 show significantly decreased S-phase arrest after ionizing radiation. However conflicting data was obtained with regards whether the formation of nibrin/Mre-11/Rad50 radiation-induced foci is affected by ATM phosphorylation of nibrin. (Lim DS *et al.,* 2000; Gatei M *et al.,* 2000; Zhao S *et al.,* 2000; Wu X *et al.,* 2000).

Mammalian Mre11 is also phosphorylated in response to ionising radiation in an ATM dependent manner; in addition, Mre11 phosphorylation is dependent on Nbs1 in human cells. (Dong ZW *et al.,* 1999).

In addition to the role of the M-R-N complex downstream of ATM in the DNA damage response pathway as discussed above, recent data also places the complex in a role upstream of ATM. Two groups have shown that the M-R-N complex is required for ATM activation and the ATM-dependent G2/M checkpoint in response to DSBs (Uziel T *et al.,* 2003; Carson CT *et al.,* 2003).

Mice with null mutations in each of the genes of the complex die early in embryogenesis. (Xiao Y and Weaver DT, 1997; Luo G *et al.,* 1999; Zhu J *et al.,* 2001).

Another feature common between AT, ATLD and NBS cells is their radiosensitivity. The nature of the molecular defect causing this radiosensitivity in NBS and ATLD cells is controversial. Initial expectation that the complex may play a role in NHEJ was due to the fact that yeast mutants with defects in *Rad50, Mre11 and Xrs2* (*NBS1* is the human functional analogue) are radiosensitive and defective in both the homologous recombination and NHEJ. (Zdzieniecka M, 1999). However, recent results indicate that Mre-complex mutants can carry out HR as efficiently as wild-type cells in most cases. Moreover clear evidence for a role of the complex in NHEJ has only been obtained in budding yeast (D' Amours D and Jackson SP, 2002). In contrast, Girard PM, *et al*, found that in noncycling NBS cells, there was a small increased fraction of unrejoined double strand breaks and, more significantly, increased chromosome breaks 24h after irradiation. Once of the cell lines (347BR) showed a nearly normal checkpoint response but marked gamma-ray sensitivity. Their conclusion was that the radiosensitivity in NBS cells is attributable to a repair defect rather than cell cycle checkpoint defects (Girard P-M *et al.*, 2000). Furthermore efficient joining of cohesive-ended DNA fragments in a cell-free assay using HeLa cell extracts required an N-M-R complex containing fraction (Huang J and Dynan WS, 2002).

1.8 MISCELLANEOUS GENES ASSOCIATED WITH SENSITIVITY TO IONISING RADIATION

1.8a Genes involved in genetic syndromes

Fanconi Anaemia (FA) is a rare autosomal recessive syndrome (incidence < 1 per 100, 000 live births) characterized by congenital abnormalities, bone marrow

aplasia and cancer susceptibility (Andrea AD and Grompe M, 2003). There is marked sensitivity to DNA cross-linking agents. Although there is a clinical impression of radiosensitivity in affected individuals (Marcou Y *et al.,* 2001; Alter BP, 2002), in-vitro studies of cellular sensitivity to ionizing radiation have revealed conflicting results (Duckworth-Rysiecki G and Taylor AM. 1985; Djuzenova CS *et al.,* 2001). In studies which have found in-vitro radiosensitivity, this has been mild (Burnet NG *et al.,* 1994) and G2-phase specific (Bigelow SB *et al.,*1979).

Seven FA related genes have been cloned and designated *FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF* and *FANCG. FANCD2* has been identified as *BRCA2* (Howlett NG *et al.,* 2002). FA proteins A, C, E, F and G form a nuclear complex which, in response to DNA damage, leads to formation of DNA-repair foci consisting of BRCA1 and BRCA2/FANCD1 (Garcia-Higuera I *et al.,* 2001). Interactions with other proteins involved in DNA damage response include the phosphorylation of FANCD2 by ATM (Taniguchi T *et al.,* 2002) and the interaction of NBS1 and FANCD2 in the repair of DNA cross-links (Nakanishi K *et al.,* 2002).

Bloom's Syndrome (BS) is another rare cancer prone syndrome with clinical features of proportional dwarfism, sensitivity to sunlight, type II diabetes, sub-/in-fertility and frequent infections (Hickson ID, 2003). BS cells are exquisitely sensitive to DNA cross-linking agents (Heartlein MW *et al.,*1987, Kurihara T *et al.,*1987) and sensitive to ionizing radiation during G2 (Aurias A *et al.,* 1985; Kuhn EM, 1980). The product of the gene responsible for this disease, *BLM*, has been identified as a member of the RecQ helicase family. Helicases separate the complementary strands of nucleic-acid duplexes in an ATP dependent manner with RecQ helicases functioning to 'repair' damaged replication forks (Hickson ID, 2003). BLM is a

phosphorylation target of ATM following gamma-irradiation (Beamish H *et al.*,2002) and is also found in RAD51 DNA damage foci (Bischof O *et al.*,2001; Wu L *et al.*, 2001).

1.8b Genes related to pro-fibrotic and inflammatory cytokines

Cytokines such as Transforming Growth Factor-beta 1 (TGF-β1) and interleukin-6 (IL-6) have been implicated in predisposition to specific radiation fibrosis and pneumonitis respectively (Andreassen CN *et al.*, 2002). TGF-beta stimulates differentiation of fibroblasts and production of extra-cellular matrix and has been shown to involved in the genesis of radiation fibrosis (Martin M *et al.*, 2000). Polymorphisms in the promoter region of the gene have been to shown to influence the plasma level of TGF-β1 (Grainger DJ *et al.*, 1999) and these polymorphisms may influence the likelihood of developing radiation fibrosis. IL-6 and interleukin-1alpha (IL-1α) are pro-inflammatory cytokines that have been implicated in the development of radiation pneumonitis; high pre-treatment plasma levels were predictive of the development of pneumonitis after thoracic radiation (Chen Y *et al.*, 2001) and both IL-1alpha and IL-6 levels were also significantly higher during, and after radiotherapy for patients who had pneumonitis (Chen Y *et al.*, 2002).

CHAPTER 2

A RETROSPECTIVE CLINICAL REVIEW OF RADIATION MORBIDITY IN PATIENTS WITH CERVIX CARCINOMA TREATED AT A SINGLE INSTITUTION

INTRODUCTION

Radiation therapy has been the main therapeutic modality for carcinoma of the uterine cervix presenting in FIGO stages IB to IV, ever since the first report of its use in this disease nearly a hundred years ago (Abbe R, 1913). Only in recent times has the addition of chemotherapy been shown to be of benefit for select subgroups of patients (Thomas GM, 1999; Monaghan J, 1999). Cure rates of 60% for FIGO stage III to 92% for FIGO stage I disease have been reported (Lanciano R *et* al., 1991). High cure rates are achievable because high doses can be delivered with a combination of external radiotherapy and intracavitary applications; the latter is able to deliver high local doses to the tumour with rapid dose fall-off to the surrounding normal tissue due to the inverse square law. Radiation technique and dose differ in different parts of the world but in general, the primary tumour and the draining pelvic lymph nodes are included in the clinical target volume. The dose to the pelvic lymph nodes and to point A have been chosen to carefully limit the morbidity to surrounding normal tissue, namely, the rectum, bladder, small intestine and vagina wall. Point A, which is where the uterine artery crosses the ureter, was chosen as a point of prescription to take into account of the most sensitive structures in the high dose part of the target volume (Dobbs J and Barrett A, 1999).

However, it is well recognised that there is inter-patient variation in radiation sensitivity as borne out by clinical studies showing significant variation between patients in normal tissue reactions to radiotherapy (Tucker SL *et al.,* 1992). This inter-individual variation is thought to be largely due to genetic differences in cellular sensitivity (Brock WA *et al.,* 1995; Burnet NG *et al.,* 1994; Johansen J *et al.,* 1996) and in spite of the

delivery of safe dose regimens, an estimated 5% of patients are at risk of injury (Busch D, 1994).

The aim of this retrospective study was to assess the incidence of severe late morbidity after curative radiotherapy for cervix cancer in a single institution and to determine whether a sensitive subgroup as described in the literature could be identified in our patient population (Table 2.6).

PATIENTS AND METHODS

PATIENTS

A retrospective review was taken of patients treated with radical radiotherapy for carcinoma of the uterine cervix from 1974 to 1988 at Edinburgh Cancer Centre.

STAGING and TREATMENT

The staging and radiotherapy treatment techniques for this cohort of patients have been described in detail (Gaze MN *et al.*, 1992, Erridge et al., 2002). Briefly, patients were staged according to the International Federation of Gynaecology and Obstetrics (FIGO) classification. Routine staging investigations included examination under anaesthesia, cystoscopy, intravenous urography and chest radiography. Before 1981, patients were treated with a 'wedge' technique. The whole pelvis was treated with anterior and posterior opposed fields with midline shielding, to give a dose of 20 Gy to the central pelvis and to point A, and 40 Gy to the pelvic sidewall in 20 fractions over 4 weeks (Brand MAJ and Kerr GR, 1982). 2 intracavitary caesium (manual afterloading) insertions delivered a further dose of 55 Gy to point A. After 1981, external radiotherapy was delivered with a three-field brick technique with no midline shield. 40 Gy in 20 fractions were prescribed (55 Gy in 30 fractions for more advanced disease). This was followed by a single intracavitary caesium insertion of 35 Gy to point A (or 27 Gy if the

higher external beam dose was delivered). From 1984, the remote afterloading Selectron system was used.

REVIEW OF CASE RECORDS

A departmental database had been set up to record the sites and severity of morbidity according to the Radiation Therapy Oncology Group (RTOG) system (Table 2.1). From this database, patients who had been recorded to have sustained severe (RTOG grade 4 and 5) morbidity 3 months or more after the start of radiotherapy treatment, that is, late injury, were identified and their case records reviewed. The mean age at diagnosis and distribution of FIGO stage in the cohort who had severe morbidity was compared to that in the whole cohort.

The site/s of severe morbidity and the mean dose at the site of the morbidity were determined. The dose at the site of morbidity was calculated from the total of the external beam and intracavitary treatment.

The median time to onset of morbidity for the entire cohort and at each of the sites was determined using the Kaplan-Meier method and compared using the log-rank test.

The incidence of morbidity at three dose ranges, <75 Gy, 75 to 85 Gy and >85 Gy at point of morbidity was assessed. In choosing these dose ranges, we judged that a dose 75 Gy to be a dose most therapists would consider safe (Perez CA *et al.,* 1984; Eifel P *et al.,* 2004). Where any individual developed more than 1 site of injury, the least dose at which injury occurred was used.

RESULTS

Patient characteristics

There were 829 patients treated radically with external beam and intracavitary radiotherapy from 1974 to 1988. The median follow-up was 10 years. Their mean age (SD) was 54.6 (12.7) years. 96 patients developed 124 episodes of RTOG grade 4 or 5 morbidity giving a crude complication rate of 11%. The mean age (SD) in this cohort of patients was 50.8 (12.7) years and the difference in mean age was statistically significant ($p<0.01$). There was no difference in the FIGO stage distribution in the whole cohort compared with the cohort of patients who developed severe late morbidity (table 2.2).

Distribution of morbidity by site, mean dose and median time to onset

There were a total of 124 episodes of RTOG grade 4 and 5 morbidity sustained by the 96 patients. The most frequent site of injury was the rectum, with 48 episodes of injury occurring in this site. This was followed by the bladder, vaginal vault, colon, small bowel, rectosigmoid colon and the pubic bone which was the site of only 1 episode of severe, late morbidity (table 2.3).

The rectum was also the most 'sensitive' structure; both the rectum and the bladder were more 'sensitive' than the vaginal wall; the mean dose (SD) delivered at the site of injury was 77.8 (20.5) Gy to the rectum, 84.2 (24.2) Gy to the bladder and 95.8 (6.3) Gy to the vaginal wall (See table 2.4 for p values). The difference between the mean dose to the rectum and that to the bladder were not statistically significant ($p=0.28$).

The median time to the onset of severe injury was 15 months for all patients (figure 2.1). The timing of complications differs according to the site of injury (table 2.4). Of note, bladder complications develop later than all other sites with a median time

to onset of 30 months compared to that of about 9 to 16 months for the other sites (p=0.015). This was also shown in the study by Perez, et al, in which 80% of rectosigmoid complications occurred within 30 months of initial therapy, in contrast to 48 months for urinary complications (Perez CA *et al.,* 1984).

Radiation dose and incidence of late injury

29 patients or 3.5% of the 829 patients treated developed injury at doses that were less than 75 Gy, 11 (1.3%) patients at between 75 and 85 Gy and 28 (3.4%) patients at doses greater than 85 Gy (table 2.5). We were unable to ascertain the dose for 28 patients.

DISCUSSION

Radiation oncologists are constantly refining treatment techniques and strategies in order to achieve maximal tumour control and cure while minimising radiation induced complications. Effective doses used for treating carcinoma of the cervix are unfortunately quite close to the tolerance doses of neighbouring organs, namely, the rectum and urinary bladder. Strategies developed over the decades to limit the morbidity from radiotherapy are many and include the use of multiple fields in the external beam component, low dose per fraction, and careful positioning of the intracavitary applicators in relation to the rectum and urinary bladder.

In this retrospective review of a cohort of 829 patients with carcinoma of the cervix treated with radical radiotherapy from 1974 –1988, we found a crude prevalence of late, severe morbidity (RTOG grade 4 or 5) of 11%. This is higher than the crude prevalence rate of 6.1% found in the recent national audit of patients treated in UK

centers in a single year, 1993 (Denton AS *et al.,* 2002). It was otherwise comparable to other studies that covered treatment periods similar to that in this study (table 2.6). Moreover in our patient population, there is a proportion (about 4%) of patients who experience severe toxicity even at doses less than 75 Gy, which would be deemed safe in most institutions (Perez CA *et al.,* 1984; Eifel P *et al.,* 2004).

There are many patient factors that may contribute to the development of late radiation morbidity (Baumann M and Bentzen SM, 2002). These include a history of smoking, presence of co-morbid conditions such as diabetes mellitus, prior surgery and concurrent chemotherapy. One of the limitations of this retrospective study is that we could not analyse the impact of these factors on the complication rates. However an analysis of stage distribution, surgery, RT type and chemotherapy in the UK national audit did not find a significant influence on complication rates (Denton AS *et al.,* 2002).

There is increasing evidence that the susceptibility of these individuals to radiation injury may be a genetic one (Brock WA *et al.,* 1995; Burnet NG *et al.,* 1994; Johansen J *et al.,* 1996). This is well recognised for patients with the rare severely radiosensitive syndromes ataxia telangiectasia and Nijmegen Breakage syndrome whose cancers are treated with a different modality or radiation schedule. West *et al* have found a spectrum of lymphocyte sensitivities in patients with carcinoma of the cervix, as measured by the surviving fraction at 2 Gy (SF2) (West CML *et al.,* 2001). They found that lymphocyte SF2 was a highly significant prognostic factor for the probability of developing late radiation morbidity. Mathematical modeling studies have suggested that as much as 80% of the observed differences in normal tissue morbidity between patients prescribed the same course of radiotherapy may be contributed by this individual difference in intrinsic radiosensitivity (Turesson I *et al.,* 1996).

Although strategies to limit morbidity from radiotherapy continue to be developed with increasing sophistication, for example, the use of intensity modulated radiotherapy, challenges remain. One such challenge is to identify the ~4% of individuals that develop severe late morbidity with a standard course of radiotherapy before treatment and tailoring treatment regimens accordingly. Elucidating the mechanisms that underlie the normal tissue radiosensitivity in these patients would pave the way for the development of rapid, robust and clinically applicable assays.

Table 2.1 RTOG LATE RADIATION MORBIDITY SCORING SCHEMA*

	0	1	2	3	4
Small/large intestine	None	Mild diarrhea Mild cramping Bowel movement 5 times daily Slight rectal discharge or bleeding	Moderate diarrhea and colic Bowel movement >5 times daily Excessive rectal mucus or intermittent bleeding	Obstruction or bleeding requiring surgery	Necrosis/ Perforation Fistula
Bladder	None	Slight epithelial atrophy Minor telangiectasia (microscopic hematuria)	Moderate frequency Generalized telangiectasia Intermittent macroscopic hematuria	Severe frequency and dysuria Severe generalized telangiectasia (often with petechiae) Frequent hematuria Reduction in bladder capacity (<150 cc)	Necrosis/ Contracted bladder (capacity <100 cc) Severe hemorrhagic cystitis
Bone	None	Asymptomatic No growth retardation Reduced bone density	Moderate pain or tenderness Growth retardation Irregular bone sclerosis	Severe pain or tenderness Complete arrest of bone growth Dense bone sclerosis	Necrosis/ Spontaneous fracture

* As it pertains to pelvic irradiation

Note:

RTOG grade 5 morbidity is defined as death directly related to radiation late effects

There is no RTOG grading for late morbidity of the vagina and in this site, grade 4 morbidity was defined as vaginal vault necrosis.

Table 2.2 FIGO DISTRIBUTION

		WHOLE COHORT (%)	PATIENTS WITH SEVERE NORMAL TISSUE MORBIDITY (%)	
NUMBER OF PATIENTS		829	96	
FIGO STAGE	Ib	197 (23.8)	19 (19.8)	p=0.2
	IIa	109 (13.1)	8 (8.3)	
	IIb	265 (32.0)	28 (29.2)	
	IIIa	15 (1.8)	1 (1.0)	
	IIIb	196 (23.6)	30 (31.3)	
	IVa	32 (3.9)	7(7.3)	

Table 2.3 DISTRIBUTION OF SEVERE MORBIDITY BY SITE

SITE OF INJURY	EPISODES OF RTOG GRADE 4-5 TOXICITY (%)
RECTUM	48 (5.8)
BLADDER	26 (3.1)
VAGINAL VAULT	22 (2.7)
COLON	13 (1.6)
SMALL BOWEL	10 (1.2)
RECTOSIGMOID	4 (0.5)
BONE	1 (0.1)
TOTAL	124

Table 2.4 RADIATION DOSE, TIME TO ONSET AND SITE OF INJURY

SITE OF INJURY	MEAN (SD) DOSE (Gy)	MEDIAN TIME (MTHS)
RECTUM	77.8 (20.5)	12
BLADDER	84.2 (24.2)	30
VAGINAL VAULT	95.8 (6.3)	15
COLON*		10
SMALL BOWEL*	rectum vs bladder p=0.281	12
RECTOSIGMOID*	bladder vs vaginal vault p=0.04	16.5
BONE*	rectum vs vaginal vault p<0.0001	9
* Dose not recorded for these sites		p=0.015

Table 2.5 RADIATION DOSE AND INCIDENCE OF LATE INJURY

DOSE DELIVERED AT SITE OF INJURY	NUMBER OF PATIENTS (%)
< 75 Gy	29 (3.5)
75 - 85 Gy	11 (1.3)
>85 Gy	28 (3.4)
Unknown	28 (3.4)

Figure 2.1

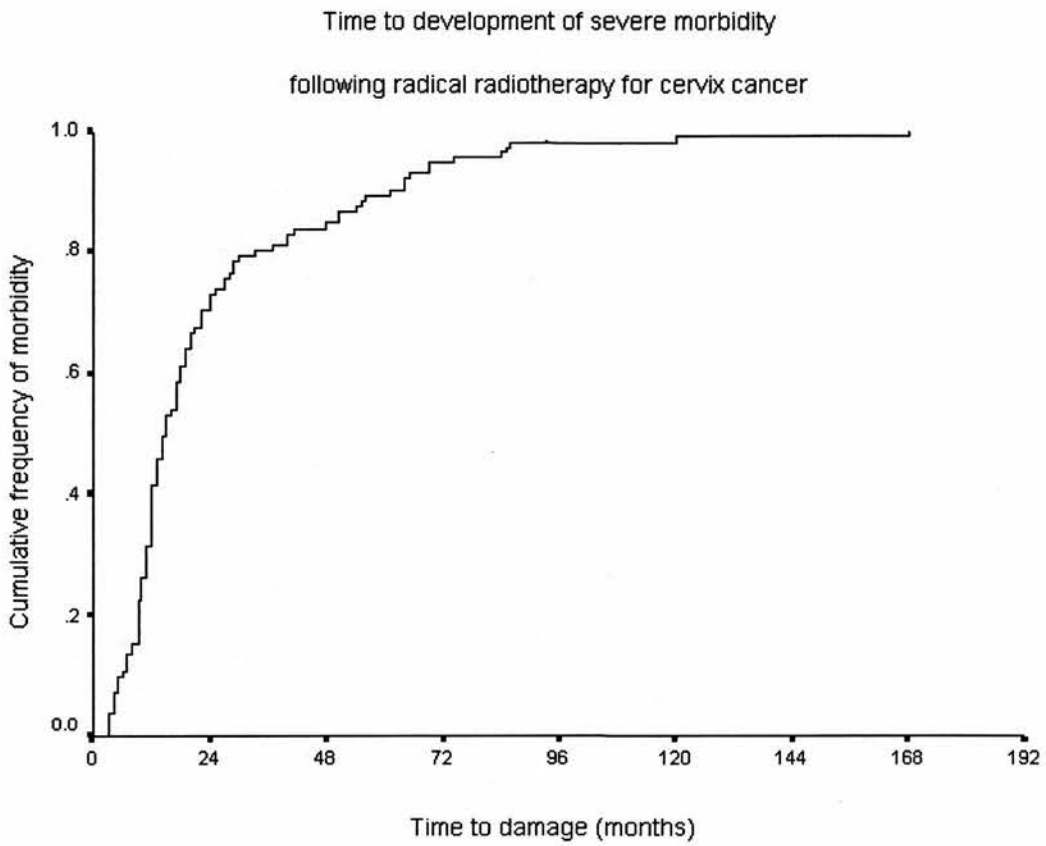

Time to development of severe morbidity
following radical radiotherapy for cervix cancer
Cumulative frequency of morbidity
1.0
.8
.6
.4
.2
0.0
0
24
48
72
96
120
144
168
192
Time to damage (months)

Table 2.6 Severe Late Morbidity After Radical Radiotherapy for Carcinoma of the Uterine Cervix

Radiotherapy Centre	Period treated	Scoring system	No. of patients	Severe Late Morbidity	Reference
National UK audit-53 centres	1993	French-Italian glossary	1558	6.1% crude ; 8 % actuarial	Denton AS *et al,* 2002
Mallinckrodt Institute of Radiology, USA	1959-1977	Similar to French-Italian glossary	811	8% grade 3	Perez CA *et al,* 1984
MD Anderson, USA	1960-1989	Similar to RTOG	1784	9.3 % at 5 years	Eifel PJ *et al,* 1995
Aarhus University Hospital, Denmark	1974-1984	AADK, Aarhus, Denmark	442	Actuarial 5 year: 28% rectosig. 13% SI 10% baldder	Pedersen D *et al,* 1994
Dr B Verbeeten Institut, Netherlands	1979 - 1991	French-Italian glossary	145	17% grade 3 1.3%grade 4	Rodrigus P *et al,* 1996
Centre de Lutte Contre le Cancer Georges-Francois Leclerc, France	1970-1994	French-Italian glossary	642	10%grade 3 3% grade 4	Barillot I *et al,* 2000

Abbreviations:

RTOG - Radiotherapy Oncology Group; Rectosig - rectosigmoid; SI – small intestines;

CHAPTER 3

MATERIALS AND METHODS

3.1 REAGENTS

General laboratory reagents were from Sigma, Fisher and BDH or as specified in the text. Molecular biology reagents were from Boeringer Mannheim unless otherwise specified. Cell culture materials were from Sigma. ATP-gamma ^{32}P was from Amersham, the phosphorimager used was by Molecular Dynamics and X-ray film was from Kodak.

3.2 PATIENTS SELECTION

A departmental database was established in the Department of Oncology in Edinburgh in 1974, and now contains data on over 70000 patients. From this registry patients treated with curative radiotherapy from 1974 until the time of recruitment were identified, who have developed RTOG grade 4 or 5 late radiation injury following treatment with standard radiotherapy doses believed to carry an acceptable risk of morbidity. We hypothesised that patients developing late injury as a result of impaired DNA repair might also be cancer-prone, and accordingly selected patients under the age of 50 years for this study. These patients had obviously also to be alive at the time of the study. Patients with necrosis rather than fibrosis were selected because we thought a cell-death endpoint was more likely to be related to a DNA repair defect than a proliferative endpoint such as fibrosis.

Local Research Ethics Committee approval was obtained for the study and the patients provided written informed consent for cell line immortalisation and the investigations. Their general practitioners and attending clinical oncologists were also informed of their patients' participation in the study.

3.3 PATIENT DETAILS

5 patients who met the above criteria agreed to participate in the study. The patients' clinical histories are summarised as follows:

Cell line **LB 0002** was derived from a patient who presented with early onset breast cancer at age 38 years and was treated by lumpectomy and adjuvant radiotherapy without systemic chemotherapy. 40 Gy in 10 fractions over 25 days were prescribed using external beam treatment followed by 42 Gy delivered by low dose-rate (0.46 Gy per hour) brachytherapy. She developed skin necrosis 14 years later. She had no recurrence 17 years post treatment.

Cell line **LB 0003** was derived from a patient who presented with early onset breast cancer at age 40 years and was treated by mastectomy and adjuvant radiotherapy without systemic chemotherapy. 45 Gy in 10 fractions over 28 days were prescribed. She developed a chest wall necrosis requiring chest wall reconstruction at 17 years, a contralateral primary breast cancer after 18 years and a presumed ipsilateral lung cancer (from which no confirmatory histology was obtained) 26 years later.

Cell line **LB0004** was derived from a patient who had a FIGO stage IB primary squamous cell carcinoma of the cervix diagnosed at age 43 years. She was a smoker with no other significant medical history. She was treated with a combination of external beam and intracavitary treatment. The dose to point A was 82 Gy. Two years following radiotherapy, she developed vesico-vaginal and ileo-vesical fistulae. Both fistulae were managed surgically and she remains disease-free 10 years later.

Cell line **LB0005** was derived from a patient who had a FIGO stage IIIB primary squamous cell carcinoma of the cervix diagnosed at age 40 years. She was a smoker with no other significant medical history. She was treated with a combination of external

beam and intracavitary treatment. The dose to point A was 80 Gy. Two years following radiotherapy, she developed a recto-vaginal fistula. This was managed surgically and she remains disease-free at 15 years post treatment.

Cell line **LB 0006** was derived from a patient who presented with early onset breast cancer at age 38 years and was treated by mastectomy and adjuvant radiotherapy without systemic chemotherapy. 44 Gy in 10 fractions over 26 days were prescribed using external beam treatment. She developed necrosis of the ipsilateral 4^{th} and 5^{th} ribs 2 years later. She has had no recurrence 17 years post treatment.

Cell line **LB0001** was derived from a 28-year old patient with a family history of breast cancer, who developed an early and sustained acute radiation reaction, but did not require cessation of therapy. She was also treated with systemic chemotherapy. No late damage has occurred, 3 years after treatment.

3.4 LYMPHOCYTE TRANSFORMATION WITH EPSTEIN-BARR VIRUS

Upon informed consent, twenty ml of venous blood from each patient were drawn into tubes containing sodium heparin (Becton Dickinson) and sent by courier to the ECACC at Salisbury, Wiltshire within the same day. The lymphocytes were extracted and transformed into lymphoblastoid cell lines with Epstein-Barr virus at the ECACC and sent back either as a frozen ampoule or a T25 flask of growing culture. Briefly the peripheral blood mononuclear cells (PBMCs) were separated by a ficoll gradient. 1 x 10^7 PBMCs were placed with 200 µl of Epstein Barr virus suspension and incubated at 37^0C for 1h. Complete RPMI feed medium (see 3.5) was then added to achieve a cell concentration of 1-2 x 10^6 cells per ml and cyclosporin A added to a final

concentration of 1 μg/ml to inactivate the T-lymphocytes. The cell suspension is incubated at 37^0C in a 5% CO_2 incubator; immortalisation takes about 4-6 weeks.

The cell lines were named at the discretion of the ECACC.

3.5 CELL CULTURE

Cell-lines were maintained at 5-8 x 10^5 cells per ml in feed medium (see below) at 37^0C in a humidified incubator gassed with 5% carbon dioxide (CM Godsen *et al.*, 1986) with change of medium 3 times a week. After two months, cell-lines were discarded and fresh stocks were defrosted from liquid nitrogen for experiments.

Cell-lines were frozen in freezing mix (see below) at a density of 1 x 10^7 per ml, kept at -80^0C overnight before transferring to liquid nitrogen for storage. On defrosting, ampoules of cells were removed from liquid nitrogen into a beaker of water at about 37^0C and the defrosted cells were immediately transferred into 20 ml of feed medium, 'washed' and centrifuged down at 700*g* for 10 minutes. They were then maintained as above.

Feed medium:

- RPMI 1640
- Foetal calf serum 10%
- Oxalacetic acid (0.1M) 0.5%
- Pyruvic acid (0.04M) 0.5%
- L-Glutamine 1.0%
- Mops buffer (1.25M) 1.0%

Freezing mix

- Dimethyl sulphoxide (DMSO) 8.0%

- Foetal calf serum 92.0%

3.6 CONTROL CELL-LINES

Control cell lines **MACSI, WALES** and **HAJON** were kindly provided by Dr Veronica Heynigen, **MDC8** by Professor Malcolm Dunlop of the MRC Human Genetics unit in Edinburgh and **SNC4** from the National Cancer Centre, Singapore. The cells of origin of all these cell lines were peripheral B lymphocytes. The former 3 cell lines were from patients with ocular abnormalities but no history of cancer while the **MDC8** was from an unaffected relative of a patient with colorectal cancer. **SNC4** was from a normal volunteer. **BD2630, GM719C** and **GM1526E** are cell lines from individuals with ataxia telangiectasia; the former was obtained from the ECACC (European Collection of Cell Cultures) and the latter two from NIGMS (National Institute of General Medical Sciences).

3.7 KARYOTYPING OF CELL-LINES

Cells were sub-cultured one day before the preparation of chromosome spreads and fed with medium to the 10ml mark in T25 flasks. Three flasks were prepared altogether. Colcemid (KayroMax at 10μg/ml Gibco Brl) was added to each flask at concentration of 1: 100 and incubated for 30, 45 and 60 min respectively at 37°C in a 5% CO_2 atmosphere. The cells were then spun down at 1200 rpm for 6 min with brake of 25%. 7.5 mls of freshly prepared 0.075 M KCl were added to the cell pellet and this was left for 10 min at room temperature. The cells were again spun down as above and the KCl decanted. The cells were then fixed three times in methanol:acetic acid at 3:1 (again freshly mixed). After the third fixation, the cells were spotted onto glass slides which had

been presoaked and cleaned in 100% alcohol to which a drop of concentrated hydrochloric acid was added.

Upon drying, the slides were sent for Giemsa staining and reading of karotype by Ms Marie Robertson and Mrs Cathy Davidson of the MRC Human Genetics Unit, Edinburgh.

3.8 RADIATION

In preparation for radiation experiments designed to assess cell viability and p53 induction, the cells were irradiated in cell culture flasks at room temperature with a calibrated linear accelerator using 6 MV photons at a dose-rate of 3.2 Gy/min. For pulsed-field gel electrophoresis experiments, a Gammacell 40 Exactor with caesium sources was used at a dose rate of 1.06Gy/min.

3.9 VIABILITY ASSAYS

Cell survival following irradiation was measured using the Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay. This is an assay based on the classical MTT cell proliferation test. The promega solution contains a tetrazolium compund [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-2(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron-coupling agent (phenazine ethosulfate; PES). The MTS tetrazolium compound is bioreduced by metabolically active cells into a coloured formazan product that is soluble in tissue culture medium. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture.

Lymphoblasts were cultured in feed medium and when exponentially growing, were irradiated at room temperature in full T25 flasks with a calibrated linear accelerator using 6 MV photons at a dose-rate 3.2 Gy per minute. The irradiation was done in the full T25 flasks instead of 96 well plates on advice from Dr David Thwaites of the Department of Oncology, Western General Hospital, Edinburgh. This was to ensure that the doses delivered would be accurate. Since 96 well plates could not be filled completely with media, the air in the plates would make the estimation of the dose delivered less accurate. Following irradiation, the cells were then plated into 96 well plates at concentrations of between 5 x 10^4 to 2 x 10^5 cells per ml. After 24, 48, 72, 96 and 108h, the cells were incubated with the 20 microlitres of the Promega solution per well at 37^0C in 5% CO_2 atmosphere ^{for} 4 hours. The plates were then read at 450 nm in a MTX Lab systems Multiskan Elisa plate reader.

Using the absorbance readings at the various time-points stated, growth curves for the cells were constructed. The vertical displacement of growth curves (Price P and McMillan TJ, 1990) compared to non-irradiated cells was then used as an estimate of the cell survival for the given dose of radiation (see figure 4.2). Two independent irradiation experiments were carried out for each cell line with at least two replicates per experiment.

3.10 CRUDE PROTEIN EXTRACT FOR IMMUNOBLOTTING

Exponentially growing cells were washed twice in PBS before lysis in approximately 2 packed cell volumes of 50mM Tris pH 7.5, 1mM EDTA, 10mM DTT, 0.2% triton X-100 and a protease inhibitor cocktail containing AEBSF, aprotinin, bestatin, E-64, leupeptin, and pepstatin A (Sigma). After incubation on ice for 30

minutes, the cellular debris was removed by centrifugation at 14000g for 15 minutes. The protein concentration of each sample was determined by the method of Bradford (Bradford MM, 1976).

3.11 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND IMMUNOBLOTTING

Aliquots (20 ul) of each sample containing equivalent amounts of protein were separated by SDS-PAGE (BioRad mini-protean) on 10% gels except for gels for DNA-PKcs and ATM blotting, for which 5% gels were used due to the large size of the proteins. Transfer to nitrocellulose membrane (Amersham) was with a semi-dry blotter at 100v for 1 hour (Owl Separation Systems, HEP-1) except for DNA-PKcs and ATM experiments, for which overnight transfer, at 30V, in a wet system (BioRad) was used. Ponceau's staining was used to confirm protein loading and transfer. After blocking with 5% milk, the membranes were probed with antibodies to Ku 70, Ku 80 and DNA-PKcs; DNA ligase IV, XRCC4, Rad 51 and ATM (Table3.1). Incubation with primary antibody was for 1 hour at room temperature or at 4^0C overnight. Following washing of the blots and incubation with the appropriate secondary antibody, detection was performed using ECL (Amersham). Unless otherwise specified, the immunoblots were performed at least twice using separate extracts in each case. The list of primary antibodies used and their working dilution are shown in Table 3.1.

3.12 WHOLE CELL EXTRACT

3.12A Exponentially growing cells were harvested, and whole cell extracts prepared according to the technique described by **Manley** (Manley JL *et al.,* 1983) and widely

Table 3.1

Acrylamide gel concentrations and primary antibodies used for immunoblotting experiments

Antibody to	Code number, source	Antibody dilution	Acrylamide gel (%)
Ku70	AHP 316, Serotec, Oxford, UK	1:4000	10
Ku80	AHP 317, Serotec, Oxford, UK	1:4000	10
DNA-PKcs	Clone 25-4, Ab-2, Neomarkers,	1:250	5
XRCC4	AHP387, Serotec, Oxford, UK	1:2000	10
DNA ligase IV	AHP554, Serotec, Oxford, UK	1;200	10
Rad51	Ab-1 #PC130, Oncogene Research Products, USA	1:1000	10
ATM (Mutated in Ataxia - Telangiectasia)	AHP392	1:1000	6
P53	Clone DO-1, Ab-6 Neomarkers, USA	1:1000	10
Retinoblastoma protein (pRB)	Clone IF8, Ab-1, Neomarkers, USA	1:250	8

Standard acrylamide:bisacrylamide ratio of 29:1 was used except for ATM gels in which an acrylamide:bisacrylamide ratio of 100:1 was utilised.

used in ***in vitro*** DNA repair systems (Wood RD *et al.*, 1988). The procedure was carried out at 4^0C. Briefly, 0.8 to 1 L of cells at a density of 0.5 - 1.0 x 10^6 cells/ml were pelleted and washed twice in phosphate-buffered saline. The cell pellet was resuspended in 4 packed cell volumes of hypotonic lysis buffer (10 mM Tris-HCl pH 8, 1 mM EDTA, 5 mM DTT and protease inhibitors) and left on ice for 20 min. The cells were disrupted by 10-20 strokes in a Dounce homogeniser, and an additional 4 packed cell volumes of 50 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 2 mM DTT, 25% sucrose and 50% glycerol added, followed by a further one packed cell volume of ammonium sulphate. After stirring for 30 min, the extract was centrifuged at 41 000 g for 3h at 4°C (Sorvall TH 641 rotor). The supernatant was removed, and 0.33 g/ml ammonium sulphate added with gentle stirring. The extract was centrifuged at 10 000 g for 30 min, after which the precipitate was recovered and resuspended in buffer containing 25 mM Hepes pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 1 mM EDTA and 17% glycerol. This was dialysed against 2 changes of the same buffer for a total of 6h, centrifuged at 10000g for 10 min, and the supernatant snap frozen and stored at -70^0C.

3.12B Whole cell extracts were also prepared according to **Baumann and West** (Baumann P and West SC, 1998) for use in cell-free plasmid DNA end-joining reactions (2.17). All procedures were at 0-4^0C. Briefly, 0.8 to 1 L of cells at a density of 0.5 - 1.0 x 10^6 cells/ml were pelleted and washed thrice in ice-cold phosphate-buffered saline and once in hypotonic lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA and 5 mM DTT). Cells were resuspended in 2 vol of hypotonic buffer for 20 min, lysed by 20 strokes in a Dounce homogeniser in the presence of protease inhibitors (Sigma mammalian cells protease inhibitor cocktail). After 20 min on ice, 0.5 vol of high salt buffer (50 mM Tris-HCl pH 7.5, 1 M KCl, 2 mM EDTA and 2 mM DTT) was added and the extract

centrifuged for 3h at 41 000g in a Sorvall TH 641 rotor. The supernatant was dialysed for 3h against buffer comprising 20 mM Tris-Hcl pH 8.0, 0.1 M KOAc, 20% (v/v) glycerol, 0.5 mM EDTA and 1 mM DTT, snap frozen and stored at -70^0C

3.13 DNA-DEPENDENT PROTEIN KINASE ASSAY

Whole cell extracts prepared as described (3.12A) were assayed for DNA-PK activity by measuring the phosphorylation of a biotinylated p53-derived peptide substrate (Promega SignaTECT DNA-Dependent Protein Kinase (DNA-PK) Assay System) in the presence or absence of calf-thymus DNA (Allalunis-Turner J *et al.,* 1995; Sirzen F *et al.*, 1999). Briefly, protein extracts were incubated with

- reaction buffer (50mM HEPES KOH, 7.5, 100mM KCl, 10 mM $MgCl_2$, 0.2mM EGTA, 0.1mM EDTA and 1mM DTT);
- biotinylated p53 derived peptide substrate;
- bovine serum albumin 0.1mg/ml;
- γ–[^{32}P] ATP mix (γ–[^{32}P]ATP and 0.1mM ATP)

in the presence (activated) or absence (control) sonicated cal thymus DNA in TE buffer. Reactions were carried out in a total volume of 25 µl for 5 minutes at 30^0C and thereafter terminated by the addition of 7.5M guanidine hydrochloride. 10 µl of terminated reaction was spotted onto squares on a biotin capture membrane which was then washed according to the manufacturer's protocol. After the membrane was dried, incorporation of γ–[^{32}P] into the peptide was analysed with a STORM PhosphorImaging system (Molecular Dynamics) using Imagequant software (confirmed by scintillation counting). Calculation of DNA-PK activity included a correction for background non-specific

kinase activity. The DNA-PK values are based on at least 3 experiments using extracts prepared on at least two separate occasions.

3.14 PULSED-FIELD GEL ELECTROPHORESIS

For the measurement of post-radiation double–strand break (DSB) repair unsynchronised cells were counted, aliquoted ($4x10^5$) and irradiated on ice with 30Gy using caesium sources (Gammacell 40 Exactor) at a dose rate 1.06Gy/min. Following irradiation fresh RPMI was added to each sample. Cells were kept in the incubator at 37° C, in 5% CO_2. Unirradiated cells and cells at each time point of repair were harvested and washed. The pellet was immediately re-suspended in 0.5% Sea Plaque low melting temperature agarose (LMP agarose) at 40°C. The cell-agarose mixture was pipetted into pre-warmed 75µl plug moulds (Bio-Rad) and allowed to set at 4°C for at least 15 minutes. To release DNA *in situ* the cell plugs were transferred to 50ml tubes containing three volumes of ice cold digestion cocktail [1% N-Lauroylsarcosine (Sigma), 0.1mg/ml Proteinase K (Finnzymes), 0.1M EDTA, 0.01M Tris-Cl, 0.02M NaCl]. Incubation was for 1h at 4°C, 2h at 50°C followed by incubation in two volumes of the same digestion cocktail containing 0.1mg/ml Proteinase K for 18h at 50°C. Plugs then were washed at room temperature in 50 volumes of TE (pH 7.6) with five changes of buffer over period of three hours, in two volumes of TE containing 40µg/ml PMSF (phenylmethylsulphonyl fluoride) for 30minutes at 50°C, in 50 volumes of TE (pH7.6) with three changes of buffer and stored in the same solution at 4°C (Sambrook, J and Russell DW, 2001)

Cell plugs were cut into samples with volume of approximately 25µl and loaded into the wells of a 0.7% agarose (Seakem Gold agarose, BMA) gel (14cm wide x 12cm long x 0.8cm thick) prepared in 0.5 x TBE (45 mM Tris, 1 mM EDTA, 45 mM boric

acid, pH 8.5). The yeast chromosome molecular weight marker from Saccharomyces cerevisiae (S. cerevisiae) was also added to each gel as control. The wells were then sealed with 0.7% LMP agarose. The PFGE unit (Bio-Rad CHEF-DRII) was filled with 2 L of 0.5 x TBE. The buffer cooling system was controlled at 10°C. The sample was run at 45V (1.7V/cm) with pulse time of 75 min for 40h, followed by 100V, 30 min pulse time, for 2h (Kiltie A *et al.*,1997).

Following electrophoresis, the gel was placed on a sheet of 3MM paper (Whatman), covered with Saran wrap (Komacs) and dried at room temperature for 30min followed by 60min at 60°C using vacuum Gel Dryer (Bio-Rad) (Kiltie AE and Ryan AJ, 1997). Then 3M paper was removed by soaking the gel in distilled water for 5-10 min, rinsed with double distilled water and placed in 150 ml SYBR Green I (BMA) nucleic acid gel stain (1:10000) in 0.5 TBE, pH8.0 at 50°C for 3h, cooled for at least 10 min on ice before analyzing using Syn Gene Gel Documentation System with Syb-100 SYBR green filter. Gel images were analyzed with Gene Tools software from Syn Gene. The fraction of damage remaining was calculated as the intensity of the lane divided by the total intensity of plug plus lane (Eastham AM *et al.*, 2001; Orton C *et al.*, 1997).

All data were corrected by subtraction of the non-specific migration detected in un-irradiated control samples. Three independent experiments were carried out for each cell line with two replicates per experiment.

The PFGE experiments were done by Dr S Korzh working with Dr Loong.

3.15 p53 INDUCTION

Exponentially growing cells were irradiated with 2 and 4Gy as described (2.8). Cells were returned to the incubator at 37^0C and harvested at the half, 1, 2, 4 and 24h

post-irradiation (Khanna KK and Lavin M, 1993) washed twice in PBS and lysed as detailed above (2.10). Immunoblotting of extracts of irradiated and control cell samples was carried out as described above using an anti-p53 antibody (clone DO-1, NeoMarkers, UK) which recognises an epitope in the N-terminus of the protein. p53 expression was quantified using densitometry (E.A.S.Y Win 32 software from Herolab GmbH Laborgeräte), and induction expressed as the ratio of irradiated to control cell values. The immunoblots were performed at least twice for each cell line with separate irradiation experiments.

3.16 pRB PHOSPHORYLATION

Exponentially growing cells flasks were irradiated with 2 and 4Gy as described (2.8). Cells were returned to the incubator at 37^0C and harvested at 2h, 4h and 22h post-irradiation (Khanna KK *et al.,* 1995). The cell pellet was washed twice in PBS lysed as detailed above (2.10). Immunoblotting of extracts of irradiated and control cell samples was carried out as described above (2.11). A 8% SDS-PAGE gel was used and the separation was at 150v for 120 min, until the 50kDa marker was near the bottom of the gel. This was found to give the best resolution between hypo- and hyperphosphorylated pRB. Transfer was with a semi-dry blotter for 1h 30 min at 300mA. Immunoblotting of extracts of irradiated and control cell samples was carried out using an anti-pRB antibody (clone IF-8, Ab-1, NeoMarkers, USA) which recognises an epitope in amino acid 703-722 of the protein. The primary antibody was used at a dilution of 1:300 and incubation was carried out for 3h at room temperature. The immunoblots were performed at least twice for each cell line with separate irradiation experiments.

3.17 CELL-FREE DNA END-JOINING

pUC18 DNA was linearised with Sma1, Pst1 and BamH1 (Boehringer Mannheim) to produce DNA with blunt ends, 3' and 5' overhangs respectively. Linearised plasmid DNA was gel purified (Qiagen) and quantified. 1 μg of plasmid DNA was incubated with 30 – 50 μg of cell-free extract at 37^0C for 30 minutes. For these experiments, whole cell extract produced according to Manley as well as according to Baumann and West were utilised. End-joining reactions were carried out in 50 mM Tris-HCl pH 7.5, 5 mM DTT, 0.1 mM EDTA, 10 mM $MgCl_2$, 2 mM ATP, 2 mM dNTPs, 25 mM phosphocreatine (Sigma) and 5 units creatininephosphokinase (Sigma) (modified from Fairman MP, 1992). Reactions were terminated by incubation with proteinase K (2mg/ml) and RNase A (1mg/ml) for another 30 min at 37^0C. After phenol-chloroform extraction and ethanol precipitation, re-joined product was run on a 1% agarose gel and visualised after staining with ethidium bromide. DNA was quantified using E.A.S.Y Win 32 software from Herolab GmbH Laborgeräte.

CHAPTER 4

RESULTS:

CELL LINE CHARACTERISTICS AND

SURVIVAL AFTER IONISING RADIATION

4.1 LYMPHOBLASTOID CELL LINES

During the course of receiving curative radiotherapy, up to 5% of individuals will be estimated to experience an unexpected, severe late morbidity (Busch D, 1994) – see chapter 1.2. Treatment and patient related factors including fractionation schedule, treatment volume, co-morbid illnesses, concomitant use of other modalities such as chemotherapy, can influence the development of radiation morbidity (Baumann M and Bentzen SM, 2002). It is also well recognised that 80- 90% of the variation in normal tissue reactions cannot be accounted for by the factors listed above and is likely due to differences in intrinsic cellular radiosensitvity of individuals (Turesson I *et al.,* 1996; Safwat A *et al.,* 2002). Thus to investigate whether the episodes of late radionecrosis in our selected patients were related to a patient predispostion to normal tissue injury, we established lymphoblastoid cell lines (LCL) from peripheral blood lymphocytes.

6 LCLs were established and used for the studies of cellular radiosensitivity and to investigate the abnormalities in the DNA damage response and DSB repair pathways. 5 of these cell lines, LB0002 to LB0006 were from patients who developed late radiation necrosis following radical radiotherapy. LB0001 was from a patient who had an early and sustained acute radiation reaction but no late morbidity. Their case histories are detailed in 3.3

We chose to use LCL for the following reasons. Lymphocytes and lymphoblastoid cell lines, from patients with the radiosensitive syndromes ataxia-telangiectasia, Nijmegen breakage syndrome and ataxia-telangiectasia-like disorder, have been shown to be radiosensitive *in vitro* (Cole J *et al.,* 1988, West CM *et al.,* 1995) and to manifest the underlying molecular defects (Carney JP *et al.,* 1998;

Varon R *et al.,* 1998; Stewart GS *et al.,* 1999). Obtaining blood is less painful than a skin biopsy and therefore more acceptable to patients. Moreover recent publications have found that for non-syndromic patients with head and neck and cervix cancers, lymphocyte sensitivity to radiation correlates well with late effects (Borgmann K *et al.*, 2002; West CML *et al.,* 2001; Hoeller U *et al.,* 2003).

4.2 CELL LINES GROWTH IN CULTURE

Once the cell lines were immortalised, they were sent back from the ECACC and their growth rates determined. At cell concentrations of 1-5 x 10^5 cells per ml of medium, whereby cells are in exponential growth, the doubling time of the newly established cell lines was between 23 to 35h (Table 4.1 and Figure 4.1); this is similar to published doubling times of 24-36h (Cole J *et al.,* 1988).

4.3 CELL LINES KARYOTYPE

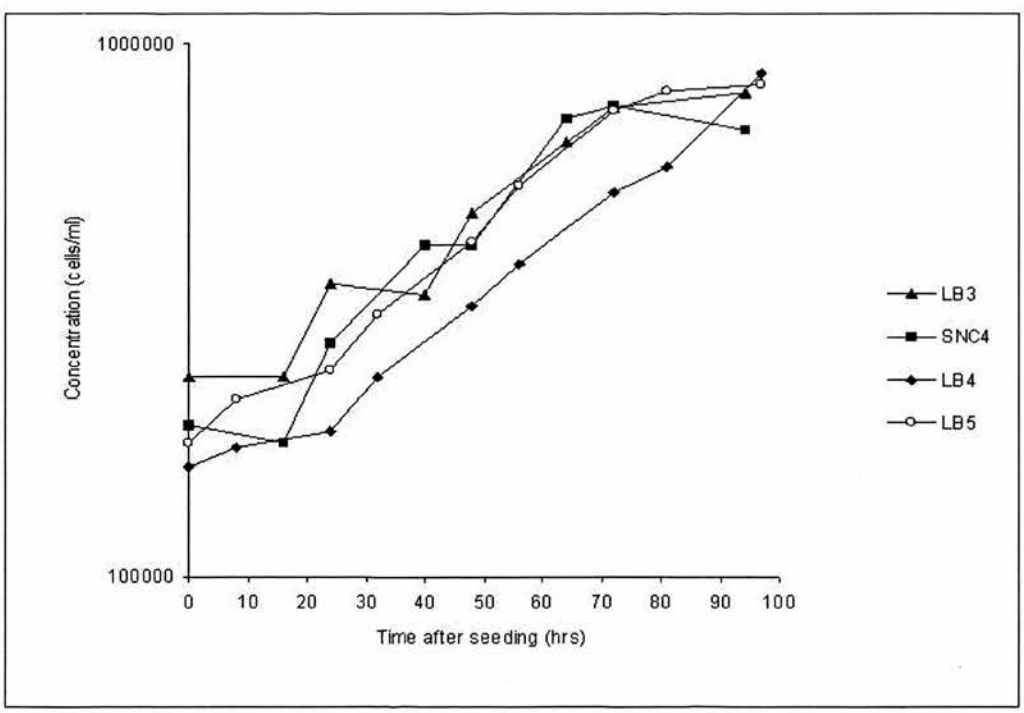
The karyotype of all 6 newly-derived cell-lines was examined using Giemsa staining of metaphase chromosomal spreads. The chromosomal spreads were prepared by the author while Giemsa staining and karyotype analysis were kindly performed by Mrs Cathy Davidson and Miss Marie Robertson (MRC Human Genetics Unit, Edinburgh, UK). All cell lines were found to be 46, XX. There were no translocations or other cytogenetic abnormalities detected (data not shown).

Table 4.1 and Figure 4.1

Cell lines growth rate and growth curves

Cells were suspended at a concentration of between 1 and 5 x 10^5 cells/ml medium (RPMI 1640, 10% FCS and additives) and incubated at 37^0C in a 5% CO_2 atmosphere. Cultures were maintained in exponential growth by subculturing every 48-72h. Regular aliquots were taken and the cell numbers were counted. The doubling time for both the control and newly derived cell lines were between 23 and 35 hours. Results are the average of at least 3 experiments per cell-line. Examples of primary growth curves for 4 of the cell lines, namely, control cell line SNC4 and newly derived cell lines LB0003, LB0004 and LB0005 are shown in figure 4.1.

Cell line	Mean Doubling-Time (h) (SE)
Macsi	23.8 (2.1)
Wales	28.0 (2.2)
SNC4	26.3 (2.1)
LB 0001	33.1 (0.7)
LB 0002	31.8 (1.2)
LB 0003	31.2 (1.0)
LB 0004	31.7 (1.1)
LB 0005	35.0 (1.1)
LB 0006	27.3 (2.0)



4.3 COMPARISON OF RADIOSENSITIVITY OF NEWLY-DERIVED, NORMAL CONTROL AND ATAXIA TELANGIECTASIA LYMPHOBLASTOID CELL LINES.

To determine whether the patients identified with late radiation injury were predisposed to this outcome due to an innate increase in cellular radiosensitivity, post-radiation viability in the EBV-immortalised lymphoblastoid cell lines was assayed, along with a number of positive and negative control lines. A viability assay was chosen because of the difficulties of carrying out colony-forming clonogenic survival assays with lymphoblastoid cells proliferating in suspension (Harrison M, unpublished). A tetrazolium compound (Promega) that is reduced by metabolically active cells was used to measure viability (Chapter 3.9). Cells in replicate 96-well plates were assayed for viability on D1, 2, 3, 5 and 7 post-irradiation. Growth curves were constructed and the vertical displacement of growth curves for irradiated cells compared to non-irradiated cells was then used as an estimate for cell survival for each given dose of radiation (Price P and McMillan TJ, 1990) (Figure 4.2). This is necessary due to the dose-dependent lag period after irradiation before regrowth is obtained; during the time that irradiated cells take to regrow (especially for the higher dose of 2 Gy), the growth rate of non-irradiated cell population would have started to plateau. Exponential extrapolation of the growth rate of the non-irradiated cells is then used to estimate cell kill (Chapter 3.9).

The number of proliferating cells after 0, 0.5, 1.0 and 2.0 Gy was determined for the cell lines LB 0002, LB0003, LB0004 and LB0006 derived from the patients with late radiation injury, the cell line LB0001, derived from an acute over-reacting patient, the negative control cell lines Macsi and Wales, and a positive control cell

line from an individual with the human radiosensitivity syndrome AT, BD 2630 (Table 4.2). These experiments showed the viability curves for LB 0002, LB0003, LB0004 and to be intermediate between those of the normal controls and an acute over-reactor on the one hand, and the AT cell line on the other (Fig. 4.3B-D). LB0001 had a viability curve that tended to be higher than the normal controls at the lowest dose of 0.5 Gy (Fig 4.3A) but similar SF_2. LB0006 had similar viability post-irradiation to the normal controls at the doses tested (Fig 4.3E).

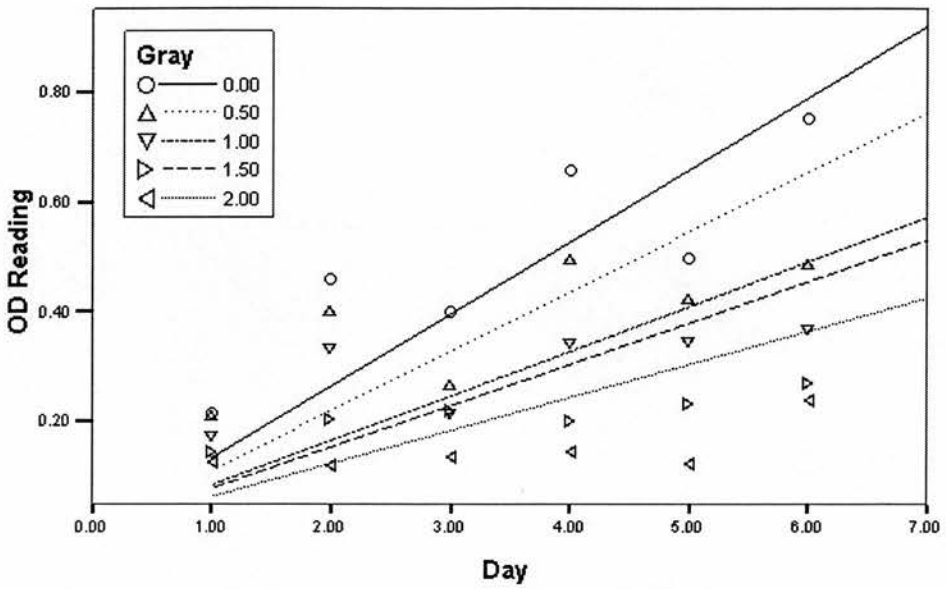
The radiation senstitivities of the cell lines can also be compared to the normal and AT cell line using the SF_2. This is the proportion of cells remaining viable after 2 Gy. The SF_2 were 0.3813 for Wales, 0.3818 for Macsi, 0.3505 for LB0001and 0.00862 for BD2630. The SF_2 for interleukin-2 dependent T-lymphocyte cell lines (Cole J *et al.,* 1988) and for normal LCLs (Angele S *et al.,*2003) is about 0.35. The mean SF_2 for lymphocytes from 83 patients with cervical carcinoma, as determined by a limiting dilution assay, was 0.33 (range 0.069 – 0.65) (West CML *et al.,* 2001).

For the cell lines from the individuals with late radiation necrosis, the SF_2 were 0.2758 for LB0002, 0.2097 for LB0003, 0.1992 for LB0004 and 0.3787 for LB0006 (See Figure 4.4). There is a modest increase in cellular radiosensitivity of LB0002, LB0003 and LB004 as measures by the SF_2. This was statistically significant only for LB0003 and LB0004 (Table 4.3). This is in keeping with the observation that cellular radiation sensitivity of phenotypically normal patients who over-react to radiotherapy can be at or just below the range for normally reacting patients (Burnet N and Peacock JH, 2002).

Figure 4.2

Post-irradiation growth curve using a proliferation assay

The *in vitro* post-radiation viability for cell line SNC 4 was determined using a proliferation assay as described in 3.9. An example of the growth curves obtained after each dose of radiation (0, 0.5, 1.0, 1.5 and 2.0 Gy) is shown. The curve for 0 Gy includes an extrapolation since the cells had reached lag phase at day 5. The vertical displacement of growth curves compared to the non-irradiated cells (0 Gy) was used as an estimate of the cell survival for the given dose of radiation.


Gray
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0.50
1.00
1.50
2.00
OD Reading
0.80
0.60
0.40
0.20
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
Day

Figure 4.3 and Table 4.2

***In vitro* radiation sensitivity of newly-derived lymphoblastoid cell lines.**

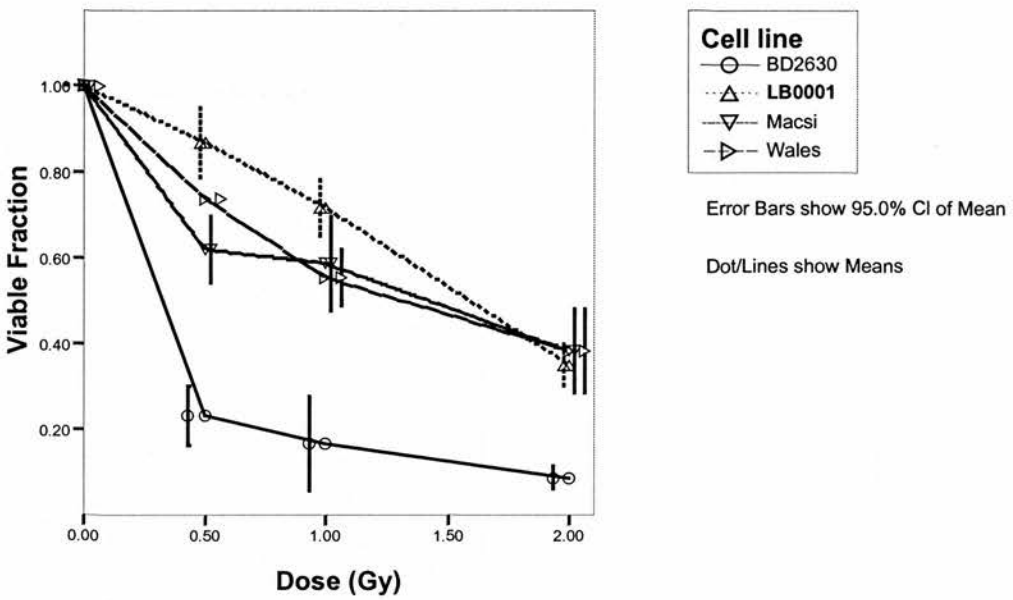
The cell line radiation sensitivity is compared against control cell lines (Macsi and Wales) and an individual with ataxia-telangiectasia (BD2630).

A. **LB0001,** derived from an individual with increased acute radiation morbidity.
B. **LB0002**
C. **LB0003**
D. **LB0004** and
E. **LB0006** are from patients with late radiation necrosis.

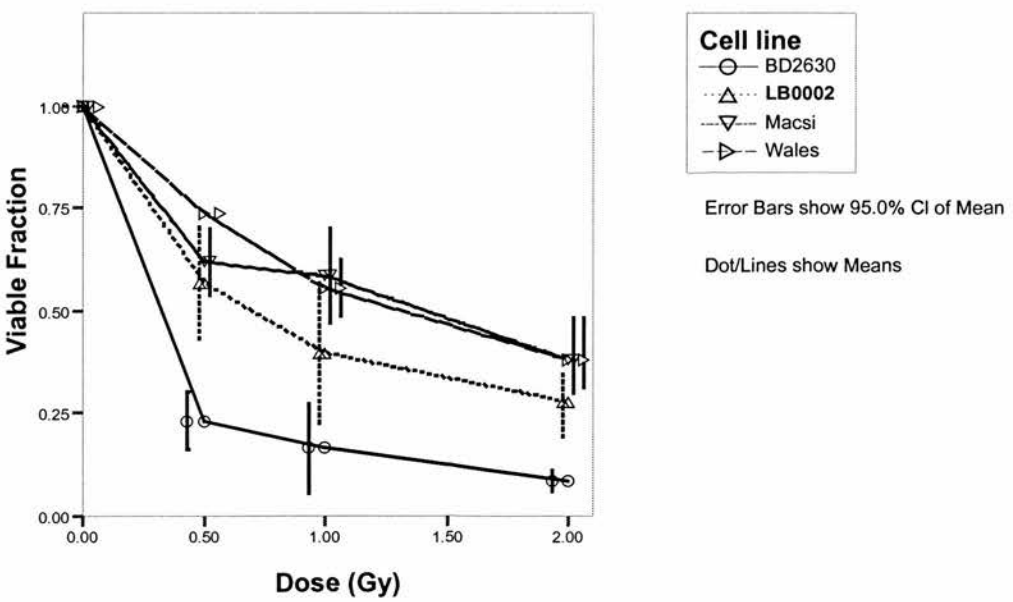
Results are from at least 3 experiments with 2 separate irradiation episodes.

Table 4.2 gives the mean viable fraction and 95% confidence intervals in numerical values from the same experiments.

A


Viable Fraction
1.00
0.80
0.60
0.40
0.20
0.00
0.50
1.00
1.50
2.00
Dose (Gy)
Cell line
BD2630
LB0001
Macsi
Wales
Error Bars show 95.0% CI of Mean
Dot/Lines show Means

B


Viable Fraction
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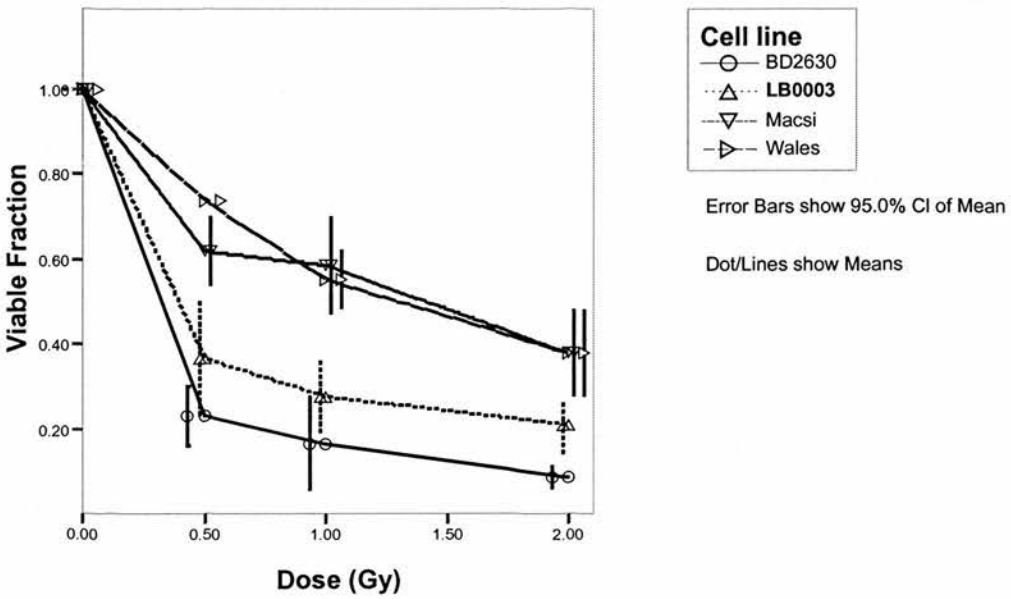
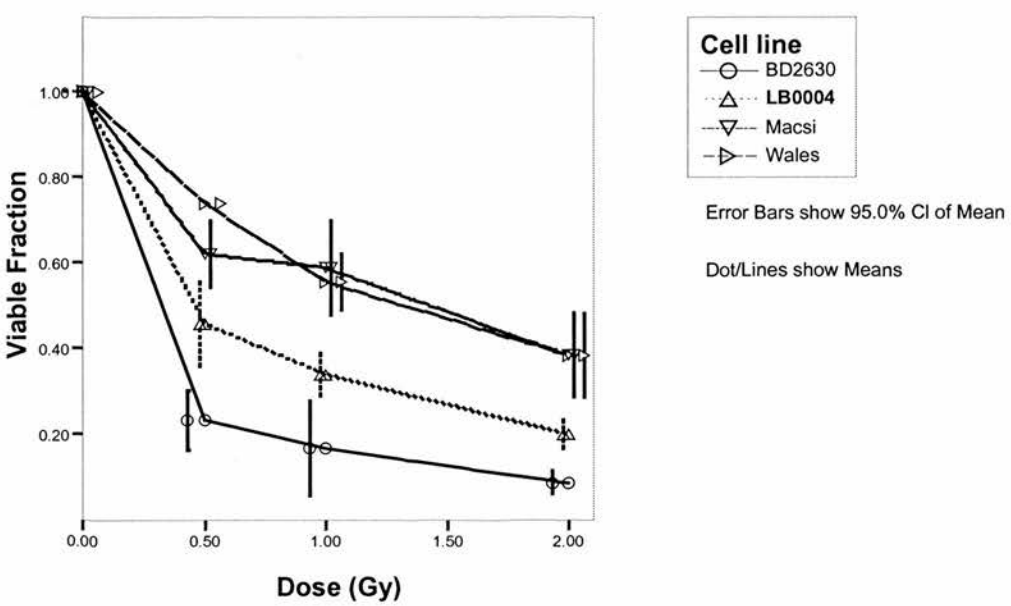
0.75
0.50
0.25
0.00
0.00
0.50
1.00
1.50
2.00
Dose (Gy)
Cell line
BD2630
LB0002
Macsi
Wales
Error Bars show 95.0% CI of Mean
Dot/Lines show Means

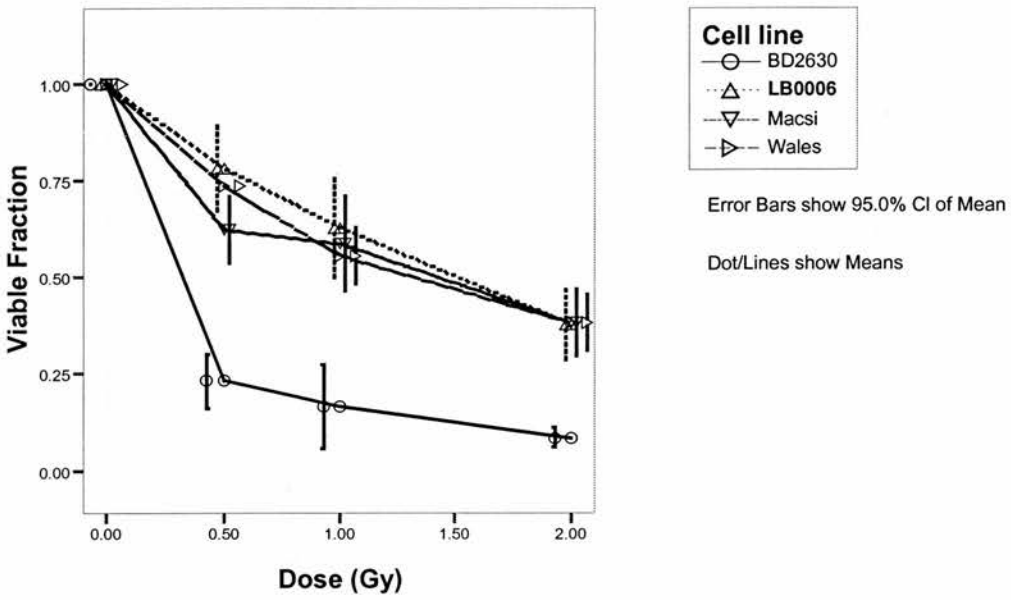
C


Viable Fraction
1.00
0.80
0.60
0.40
0.20
0.00
0.50
1.00
1.50
2.00
Dose (Gy)
Cell line
BD2630
LB0003
Macsi
Wales
Error Bars show 95.0% CI of Mean
Dot/Lines show Means

D


Viable Fraction
1.00
0.80
0.60
0.40
0.20
0.00
0.50
1.00
1.50
2.00
Dose (Gy)
Cell line
BD2630
LB0004
Macsi
Wales
Error Bars show 95.0% CI of Mean
Dot/Lines show Means

E


Viable Fraction
1.00
0.75
0.50
0.25
0.00
0.00
0.50
1.00
1.50
2.00
Dose (Gy)
Cell line
BD2630
LB0006
Macsi
Wales
Error Bars show 95.0% CI of Mean
Dot/Lines show Means

Cell line	**Mean Viable Fraction** after **0.5 Gy**	**95% confidence intervals**
Macsi	0.62	0.59-0.66
Wales	0.74	0.71-0.76
LB0001	0.87	0.83-0.90
LB0002	0.57	0.51-0.62
LB0003	0.37	0.31-0.42
LB0004	0.46	0.42-0.50
LB0006	0.78	0.73-0.83
BD 2630	0.23	0.21-0.26
	after **1.0 Gy**	
Macsi	0.59	0.54-0.64
Wales	0.56	0.46-0.57
LB0001	0.72	0.69-0.75
LB0002	0.40	0.33-0.47
LB0003	0.28	0.24-0.31
LB0004	0.34	0.32-0.36
LB0006	0.63	0.57-0.68
BD 2630	0.17	0.13-0.25
	after **2.0 Gy**	
Macsi	0.38	0.35-0.42
Wales	0.38	0.36-0.41
LB0001	0.35	0.33-0.38
LB0002	0.28	0.24-0.31
LB0003	0.21	0.18-0.24
LB0004	0.20	0.18-0.21
LB0006	0.38	0.34-0.42
BD 2630	0.086	0.077-0.095

Figure 4.4

Surviving fraction at 2 Gy (SF_2)

A comparison of the SF_2 of lymphoblastoid cell lines from patients with late radionecrosis (LB0002-0006), an individual with increased acute radiation morbidity (LB0001), an individual with ataxia telangiectasia (BD2630) and control cell lines (Macsi and Wales). A proliferation assay was used to ascertain the SF_2. Bars represent the mean from at least 3 experiments from 2 separate irradiation episodes. Error bars represent mean +/- 1 standard error.

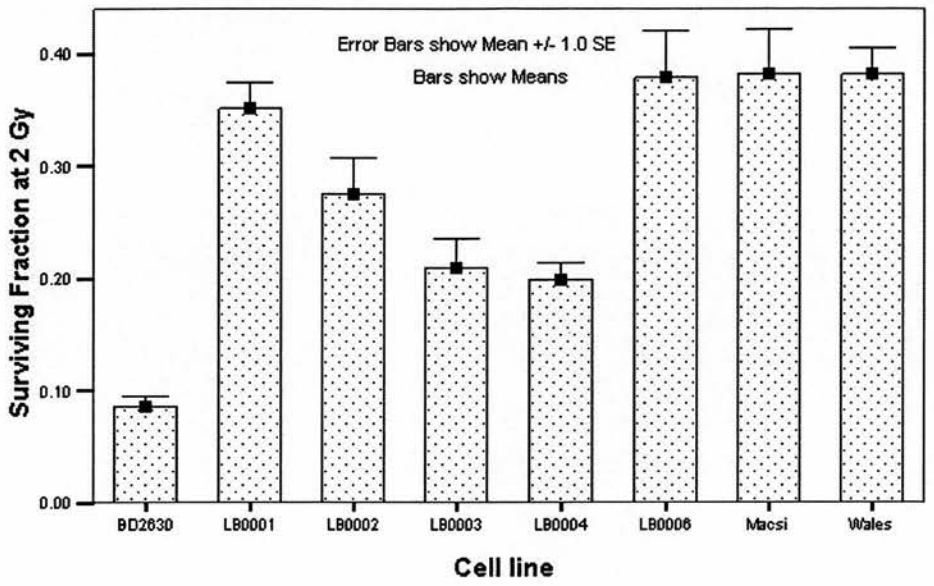

Error Bars show Mean +/- 1.0 SE
Bars show Means
Surviving Fraction at 2 Gy
0.40
0.30
0.20
0.10
0.00
BD2630
LB0001
LB0002
LB0003
LB0004
LB0006
Macsi
Wales
Cell line

Table 4.3

Mean viable fractions of the newly derived cell lines compared with control cell lines

The mean viable fraction after 0.5, 1.0 and 2.0 Gy ionising radiation is compared against control cell lines Macsi and Wales and BD2630, a cell line from an individual with ataxia telagiectasia. The p values are obtained using t-test for the comparison of means. NS = non-significant at the 0.05 level.

	LB0001	LB0002	LB0003	LB0004	LB0006
O.5 Gy					
Macsi	NS	NS	**p=0.003**	**p=0.014**	NS
Wales	NS	NS	**p=0.003**	**p=0.002**	NS
BD2630	NS	NS	NS	NS	NS
1.0 Gy					
Macsi	NS	NS	**p=0.001**	**p=0.001**	NS
Wales	NS	NS	**p=0.002**	**p<0.0001**	NS
BD2630	NS	NS	NS	NS	NS
2.0 Gy					
Macsi	NS	NS	**p=0.005**	**p=0.002**	NS
Wales	NS	NS	**p=0.004**	**p<0.0001**	NS
BD2630	NS	NS	NS	NS	NS

When fibroblasts from 130 non-syndromic breast cancer patients were evaluated for cellular radiosensitivity, the D0.01 (the dose required to reduce the surviving fraction too1%) was found to distribute along a normal distribution with 6 Gy being the mean D0.01. Fibroblasts from severe over-reactors (Burnet N *et al.,* 1998) were found to have D0.01 of 4.5 Gy (about 20% less than the mean). This was in contrast to fibroblasts from extreme over-reactors, which include AT patients and a non-syndromic individual form whom 180BR (which has a Lig4 defect) for example, the D0.01 for 180BR is 2.39. In the study by Ramsey and Birrell, they too found a statistically significant difference in the SF_2 for LCLs from non-syndromic breast cancer patients manifesting different grades of radiation morbidity. The SF_2 for patients with grade 2-4 was 0.12 compared with 0.18 for those with grade 0 or 1 reactions (Ramsay J and Birrell G, 1995); this represents a difference of about 33%. The SF_2 for the AT cell line in this study was 0.01.

Surprisingly, LB0006 had cellular sensitivities no different from the normal controls. This would suggest that factors other than cellular sensitivity account for the patient's clinical response to radiotherapy (Marcou Y *et al.,* 2001). Interestingly, the patient from whom LB0006 was derived had bone necrosis as opposed to the soft tissue necrosis that the other patients sustained. This might suggest that severe bone reactions from radiation may have different underlying mechanisms than soft tissue reactions and that factors other that intrinsic cellular sensitivity are important.

Despite three attempts, obtaining viability data was not successful for cell line LB0005 which was also derived from a patient with late radiation necrosis. This was primarily due to the difficulty of maintaining the cell-line in culture in 96 well

plates for the period of time it requires (4 to 7 days) to do the viability assays using this method.

In conclusion, 2 of the lymphoblastoid cell lines (LB0003 and LB0004) established from patients with late radionecrosis were significantly more sensitive to radiation *in vitro*, compared with control cell lines (Macsi and Wales) as well as with the other newly derived cell lines LB0001, LB0002 and LB0006.

CHAPTER 5

RESULTS:

INVESTIGATION OF DNA DOUBLE-STRAND BREAK REPAIR IN THE NEWLY-DERIVED CELL LINES

DNA double-strand breaks are the major lethal cellular events following exposure to ionising radiation. A number of different models of double-strand break repair have been described (Chapter 1 – Introduction, sections 1.6a and 1.6b). Mammalian cells utilise both homologous recombination (HR) and non-homologous end-joining (NHEJ) to repair DNA DSBs, with the major pathway being NHEJ. The predominant process appears to be dependent on cell type, stage of development or phase of the cell cycle. In G1/G0 phase, the great majority of DSBs are rejoined by NHEJ (Lee SE *et al.,* 1997; Takata M *et al.,* 1998). Homologous recombination in mammals is important during the S- and G2-phases of the cell cycle where optimally positioned sister chromatids present a perfect template for exchange (Hendrickson EA, 1997; Takata M *et al.,* 1998; Sonoda E *et al.*, 1998).

We hypothesised that patients who develop late necrosis after doses of radiation that are tolerated by the majority of individuals may be predisposed by a cellular defect in DNA DSB repair. Accordingly we investigated the presence and activity of the key NHEJ proteins and of a key component of the HR pathway, Rad51, in our cell-lines LB0002-LB0006. We also studied the capacity and kinetics of selected cell-lines to repair DNA DSBs by pulsed-field gel electrophoresis.

5.1 WESTERN BLOT ANALYSIS OF PROTEINS INVOLVED IN DOUBLE-STRAND BREAK REPAIR

The factors involved in NHEJ pathway that have been identified are the Ku-70, Ku-80 and the catalytic sub-unit of DNA-PK (these from a heterotrimer DNA-PK); XRCC4 and DNA ligase IV and more recently the Artemis protein (section 1.6b). At the time of this study, Artemis had not been discovered. Animal models in which components of the NHEJ pathway are deficient are radiosensitive. In humans, M059J, a

radiosensitive glioma cell line lacks DNA-PKcs expression (Lees-MillernSP *et al.*, 1995). Extreme radiosensitivity in a number of paediatric patients has now been described in association with hypomorphic mutations in DNA ligase IV (O'Driscoll M *et al.*, 2001).

We hypothesised that patients contributing LB0002-LB0006 might have been predisposed to their radiation morbidity due to a biochemical abnormality in the NHEJ proteins. Accordingly immunoblots were performed to the proteins that were known to be involved in the non-homologous end-joining (NHEJ) process. In NHEJ the DNA dependent protein kinase (DNA-PK) heterotrimer localise to the DNA ends of a DSB. Ku70 and Ku80 first bind to DNA ends and in turn recruit DNA-PKcs. The immunoblots for each of these sub-units showed that the proteins were present in equal amounts and at the expected molecular weights in all the cell lines (Figure 5.1A-C).

Two other proteins considered important in NHEJ are DNA ligase IV and XRCC4. Western blots of DNA ligase IV and XRCC4 show that both proteins are present in all of the cell-lines (Figure 5.2).

Rad51 is a key enzyme involved in HR, an alternative pathway by which mammalian cells, especially in S- and G2-phases of the cell cycle, repair DNA DSBs. Immunoblotting for rad51 showed that it was also present in equal quantities in all the cell lines (Figure 5.3).

5.2 DNA-PK ACTIVITY

A radiosensitive CHO cell-line XR2, belonging to the XRCC7 complementation group, is known to have normal expression of DNA-PKcs. This is associated with reduced kinase activity of the enzyme. More recently, this was attributed to a missense

Figure 5.1

Western blotting for the presence of Ku70, Ku80 and DNA-PKcs

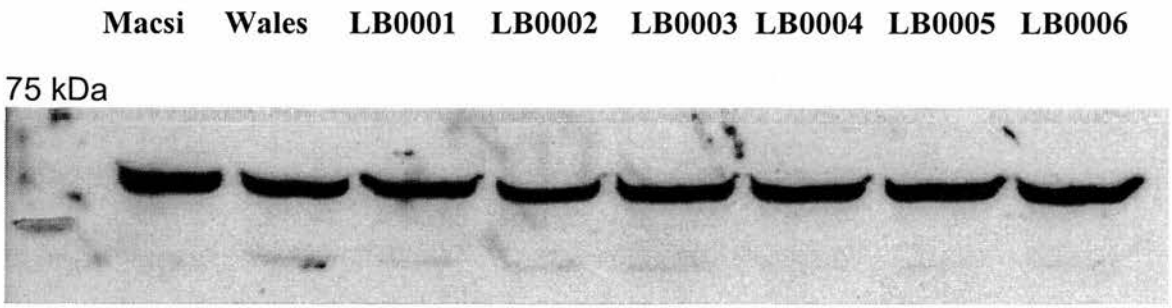
(A) and (B)

25 μg of whole cell extracts from control cell lines Macsi and Wales and each of the newly derived cell lines LB0001-LB0006 were loaded onto a 10% SDS-polyacrylamide gel and the proteins separated by electrophoresis. The proteins were transferred onto nitrocellulose membrane and then probed with antibodies to Ku70 and Ku80.

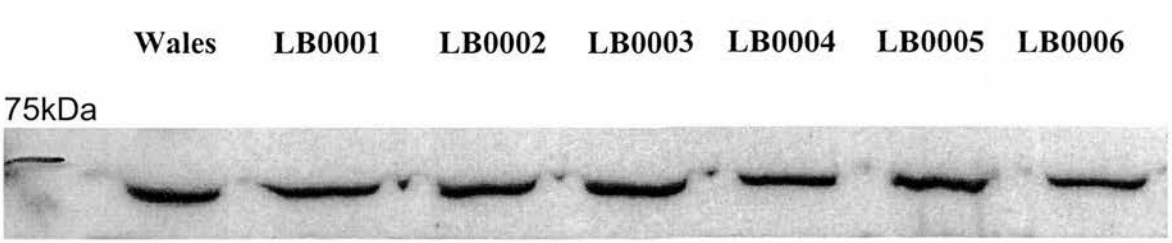
All the newly derived cell lines showed equal amounts of both Ku80 **(A)** and Ku70 **(B)**.

(C) The same procedure was applied except that 100 μg of whole cell extract was utilised and separation was on a 5% SDS-polyacrylamide gel. The transferred proteins were probed with antibody to DNA-PKcs. All the newly derived cell lines showed equal amounts of DNA-PKcs **(C)**.

A


Macsi
Wales
LB0001
LB0002
LB0003
LB0004
LB0005
LB0006
75 kDa

B


Wales
LB0001
LB0002
LB0003
LB0004
LB0005
LB0006
75kDa

C

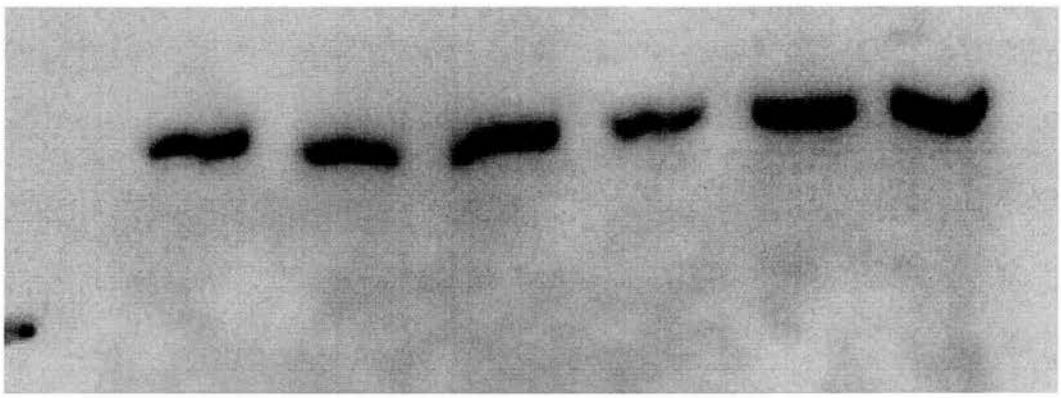


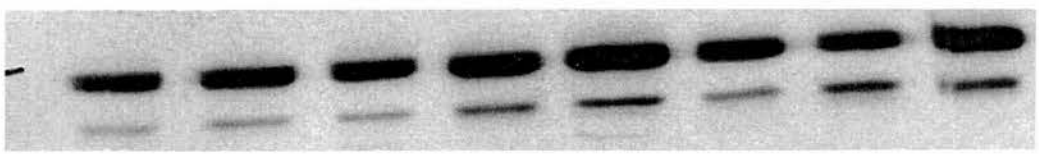
Figure 5.2

Western blotting for the presence of XRCC4 and DNA ligase IV

30 μg of whole cell extracts from control cell lines Macsi and Wales and each of the newly derived cell lines LB0001-LB0006 were loaded onto a 10% SDS-polyacrylamide gel and the proteins separated by electrophoresis. The proteins were transferred onto nitrocellulose membrane and then probed with antibodies to XRCC4 and DNA ligase.

All the newly derived cell lines showed equal amounts of both XRCC4 **(A)** and DNA ligase IV **(B)**. The phosphorylated (upper band) and unphosphorylated (lower band) forms of XRCC4 were present in all the cell lines.

A


50 KDa
Macsi
Wales
LB0001
LB0002
LB0003
LB0004
LB0005
LB0006

B

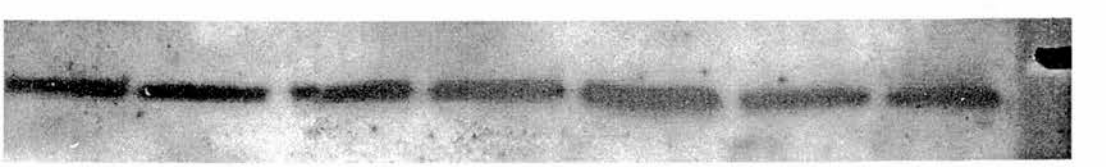
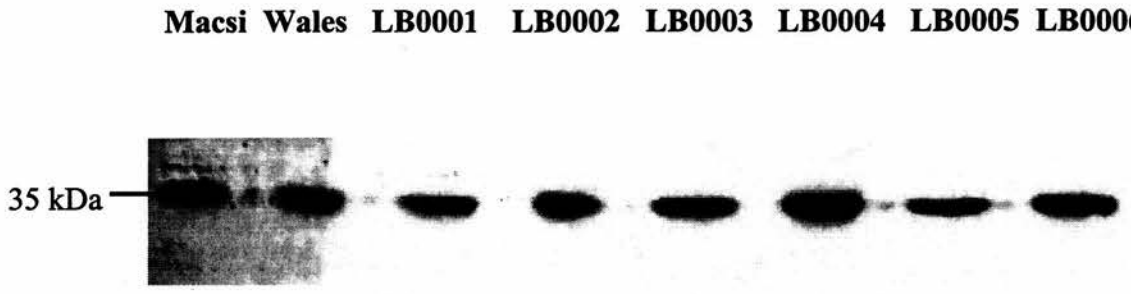

LB0001
LB0002
LB0003
LB0004
LB0005
LB0006
Macsi
105 kDa

Figure 5.3

Western blotting for the presence of Rad51

30 μg of whole cell extracts from control cell lines Macsi and Wales and each of the newly derived cell lines LB0001-LB0006 were loaded onto a 10% SDS-polyacrylamide gel and the proteins separated by electrophoresis. The proteins were transferred onto nitrocellulose membrane and then probed with antibodies to Rad51.

All the newly derived cell lines showed equal amounts of Rad51.


Macsi Wales LB0001 LB0002 LB0003 LB0004 LB0005 LB0006
35 kDa

mutation that resulted in an amino acid substitution of glutamic acid for glycine 6 residues from the C-terminus of the DNA-PKcs protein (Woods T, *et al.,* 2002).

We hypothesised therefore that LB0002-LB0006 might have decreased DNA-PK activity despite normal expression of all three subunits and accordingly DNA-PK activity was assayed in the cell lines. To assay for DNA-PK activity, DNA containing strand termini and all 3 subunits of the complex are required (Finnie NJ, *et al.*, 1995). The phosphorylation of the DNA-PK peptide substrate in the presence of calf thymus DNA was measured to correct for any background phosphorylation (in the absence of calf thymus DNA) by non-specific kinases.

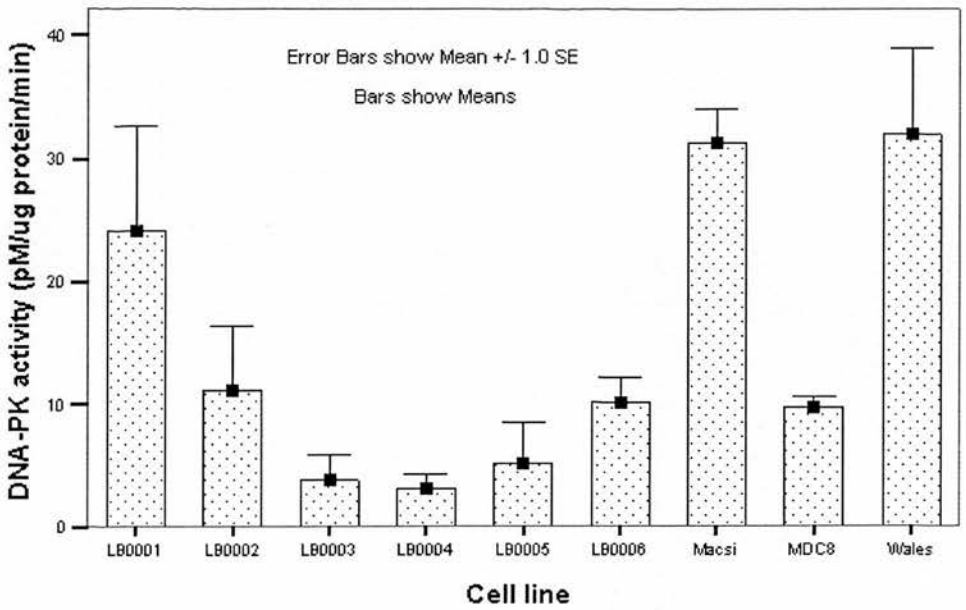
A statistically significant reduction of DNA-PK activity was observed in two of the cell-lines, LB0003 and LB0004 compared to Macsi, Wales, MDC 8. Compared to all three control cell lines, LB0004 had a 3-10 fold and LB0003 a 2.5-8 fold reduction in DNA-PK activity. LB0001, LB0002, LB0005 and LB0006 demonstrated levels of DNA-PK activity that were not significantly from the control cell lines (Figure 5.4 and Tables 5.1A & B).

As the cellular phenotype of LB0003 and LB0004 are similar to XR-C2, it was hypothesised that a missense mutation in the catalytic domain of DNA-PKcs may also be found in one or both of these cell-lines. Sequencing for the analogous mutation in the catalytic domain of DNA-PKcs in the two cell-lines has been done and neither LB0003 nor LB0004 contains the mutant sequence (data not shown). Threonines 2637 and 2647 of DNA-PKcs are autophosphorylation sites that are essential for cellular resistance to ionising radiation; substitution of these residues by alanine renders cells radiosensitive (Soubeyrand S *et al.,* 2003). Sequencing confirms that both LB0003 and LB0004 contain

Figure 5.4 and Tables 5.1A, 5.1B

DNA-PK activity in the cell lines

Peptide phosphorylation by cell-free extracts were determined in the presence and absence of calf thymus DNA termini. Data are the mean of at least 3 separate experiments. Error bars show mean +/- 1 SE. LB0003 and LB0004 showed significantly less DNA-PK activity compared to the control cell lines. The ratio of mean DNA-PK activity of control cell lines Macsi, Wales and MDC8 to that of the LB0001-LB0006 cell lines are shown in table 5.1A. The p values obtained from comparing the mean DNA-PK activities are shown in table 5.1B.



5.1A

	Ratio of DNA-PK Activity					
	LB0001	LB0002	LB0003	LB0004	LB0005	LB0006
Macsi	1.29	2.80	**8.24**	**10.03**	6.10	3.06
Wales	1.32	2.87	**8.42**	**10.25**	6.23	3.13
MDC8	0.40	0.88	**2.57**	**3.13**	1.90	0.96

5.1B

	Comparing Mean DNA-PK Activity					
	LB0001	LB0002	LB0003	LB0004	LB0005	LB0006
Macsi	p=0.331	p=0.004	p<0.001	p<0.001	p=0.001	p=0.001
Wales	p=0.525	p=0.084	p=0.021	p=0.019	p=0.054	p=0.061
MDC8	p=0.187	p=0.812	p=0.015	p=0.001	p=0.309	p=.823

threonine residues at 2637 and 2647 (data not shown). At the present time, the underlying mechanism for the reduced DNA-PK activity is still unknown.

5.3 PULSE FIELD ANALYSIS OF DOUBLE STRAND BREAK AND REPAIR

This work was undertaken following the *in-vitro* DNA-PK assays and the technical work performed by Dr Svitlana Korzh. We expected that cell lines with decreased DNA-PK activity would demonstrate impaired DNA DSB repair (Nevaldine B *et al.,* 1997; DiBiase SJ *et al.,* 2000) and sought to confirm this in LB0003 and LB0004, which were examined for their ability to repair DNA DSBs using pulse field gel electrophoresis. Macsi and LB0001 were used as controls.

Pulse field gel electrophoresis is a sensitive and specific technique that allows for the measurement of the initial amount of DNA DSBs induced by agents such as ionising radiation. In addition it also allows for the observation of the rate and total amount of double-strand break repair. In brief, cells are irradiated and sequential cell aliquots are taken at fixed time-points after irradiation. The cells are embedded in agarose plugs and subject to incubation in buffers that would lyse the cell membranes. The plugs containing the genomic DNA are subject to pulsed field electrophoresis. Genomic DNA is so long that it cannot enter into the agarose gel matrix from the wells of the gel. However when double –strand breaks have been induced, the DNA fragments are now small enough to migrate out of the wells into the gel matrix. The amount of DNA that is in the gel outside the well, is expressed as a fraction of the total amount of DNA in the entire lane, that is, inside the well and in the gel (fraction of DNA 'released'). This is taken to represent the quantity of double-strand breaks that have occurred

With time, the cells repair the DSBs and the DNA regains its initial size and can no longer enter the gel. The rate at which the cell repairs DSBs can then be observed by

measuring the fraction of DNA released over time after a given dose of ionising radiation. At the end of the repair period, the residual quantity of DSBs remaining can also be ascertained.

The initial induction of DSBs was equivalent in the 4 cell-lines across the doses tested (Figure 5.5A). Approximately 60% of the DSB that had been observed at 0 min were repaired by the control cell-lines by 30 min, whereas only 5% were repaired by LB0003 and LB0004 within the same 30 min period (Figure 5.6B and Table 5.2). Although this early difference in DSB rejoining had been partly reversed by 4h, the number of residual DSB at 24h was higher in LB0003 and LB0004 than Macsi and LB0001. In short, the two radiosensitive cell lines with deficient DNA-PK activity do appear to exhibit impaired DNA double-strand break repair, particularly in the fast component.

5.4 DNA END JOINING ASSAY

Genetic studies have identified the proteins involved in non-homologous end joining (NHEJ), which is the main repair mechanism for DNA double strand breaks in higher eukaryotes. Biochemical approaches such as *in-vitro* cell-free NHEJ systems could address questions that are difficult to answer with genetic studies (Labhart P, 1999). Questions such as, what is the minimal number of proteins required for the joining of specific DNA end structures? Is the involvement of genetically identified proteins in NHEJ direct or indirect? What enzymatic activities are present among the NHEJ factors and what is their role in NHEJ or in its regulation?

Since ionising radiation inflicts most of its damage via the production of DNA double strand breaks and the main repair mechanism for these are NHEJ, the aim of these

Figure 5.5

DNA double-strand break (DSB) induction as measured by pulsed field electrophoresis (PFGE) assays.

Cells from cell lines Macsi, LB0001, LB0003 and LB0004 were exposed, on ice, to doses of ionising radiation as shown. Cells were immediately subject to lysis and then PFGE applied to the genomic DNA. Results shown are the mean of at least 3 separate samples per cell line. Error bars represent mean +/- 1 SE. Data are corrected by the subtraction of non-specific DNA migration detected in unirradiated samples. There was no difference in the amount of DNA DSBs induced by a given dose of irradiation (from 2.5 to 80 Gy) between the different cell lines tested.

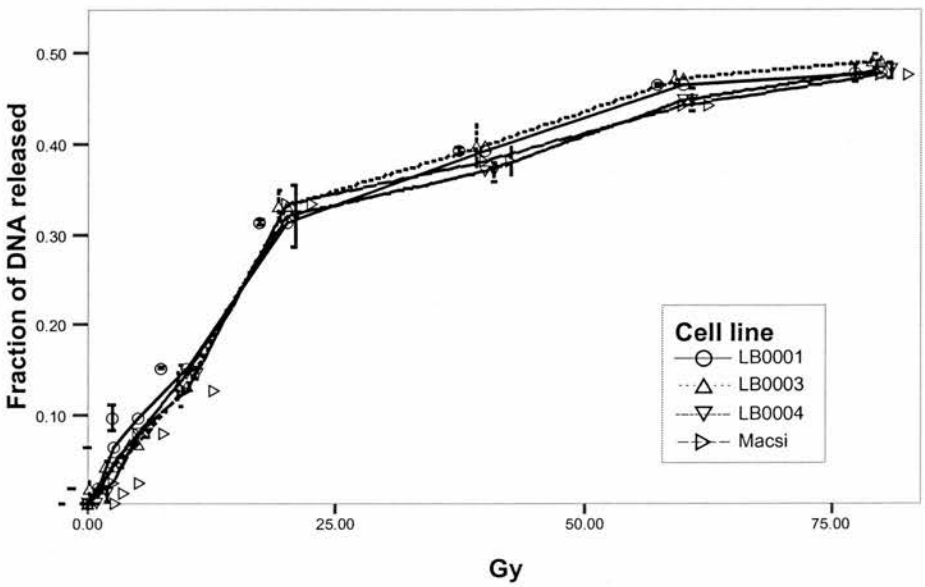
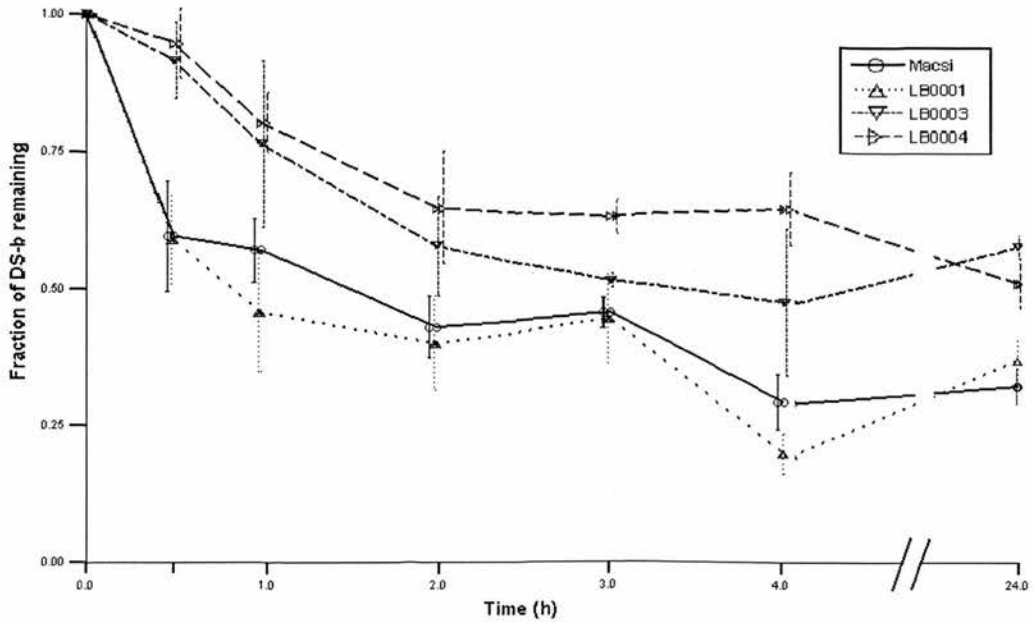

Fraction of DNA released
0.50
0.40
0.30
0.20
0.10
0.00
25.00
50.00
75.00
Gy
Cell line
LB0001
LB0003
LB0004
Macsi

Figure 5.6 and Table 5.2

DNA double-strand break (DSB) rejoining as measured by pulsed field electrophoresis (PFGE) assays.

Cells from cell lines Macsi, LB0001, LB0003 and LB0004 were exposed, on ice, to 30 Gy of ionising radiation and returned to the incubator at 37^0C, 5%CO_2. At the time-points shown, aliquots of cells were lysed and the genomic DNA subject to PFGE. Results shown are the mean of at least 3 separate samples per cell line. Error bars represent mean +/- 1 SE. Data are corrected by the subtraction of non-specific DNA migration detected in unirradiated samples. The cell lines with reduced DNA-PK activity, LB0003 and LB0004 showed significantly less rejoining of DNA DSBs at 0.5h compared with Macsi and LB0001.



5.2

	Mean Fraction of DNA DSB remaining			
	Macsi	**LB0001**	**LB0003**	**LB0004**
Time after IR (H)				
0.5	**0.59** (0.54-0.64)	**0.59** (0.54-0.64)	**0.91** (0.87-0.95)	**0.95** (0.91-0.99)
1	**0.57** (0.53-0.61)	**0.45** (0.38-0.52)	**0.76** (0.67-0.85)	**0.8** (0.76-0.84)
2	**0.43** (0.4-0.46)	**0.4** (0.35-0.45)	**0.58** (0.52-0.64)	**0.65** (0.58-0.72)
3	**0.45** (0.43-0.47)	**0.44** (0.39-0.49)	**0.51** (0.5-0.52)	**0.62** (0.6-0.64)
4	**0.29** (0.26-0.32)	**0.2** (0.18-0.22)	**0.47** (0.39-0.55)	**0.64** (0.6-0.68)
24	**0.32** (0.3-0.34)	**0.37** (0.34-0.4)	**0.58** (0.57-0.59)	**0.51** (0.48-0.54)

experiments was to develop a robust *in-vitro* cell-free system dependent on factors involved in NHEJ. Such a system could be used to assay cell free extracts from patients who are scheduled to receive radiotherapy, for the ability of the former to rejoin DNA DSBs by NHEJ. The intention was to subsequently develop the system as a predictive biochemical assay for normal tissue intrinsic radiosensitivity.

In the presence of cell free extracts, from control cell lines (for example, Macsi), prepared according to Manley (see chapter 3.12A), linearised pUC18 DNA was observed to form linear dimers and occasionally trimers and higher multimers. There was high variability of the degree of end joining under the reaction conditions described and the rest of the data obtained in these experiments must be considered preliminary only. Nevertheless, preincubating the extracts with antibodies to Ku 70, Ku 80, DNA-PKcs and DNA ligase IV did not inhibit end-joining of the linearised plasmid DNA. There was also no inhibition in the presence of 10 and 50 mM LY2940002, a small molecule inhibitor of DNA-PKcs kinase activity.

Experiments using the method of cell-free extract preparation of Baumann and West, (Baumann P and West SC, 1998b) were also performed. This was the first description (and the only one at the time of this study) of an *in-vitro* system for NHEJ that is dependent on the subunits of DNA-PK and DNA ligase IV. Very little repair product was observed using the method of cell free extract preparation described (data not shown). However the protein concentration of repeated extracts from different control and the newly derived cell lines was substantially below the 15 mg/ml described as necessary for joining activity (personal communication, Dr P Baumann).

One possibility for the failure of our experiments using the Manley extracts, to demonstrate dependence of end-joining on the NHEJ factors could be related to the

reaction conditions used for the end-joining per se. We carried out the end-joining reactions in the presence of 10 mM $MgCl_2$ (chapter 3.17) whereas the reaction of Baumann and West used 0.5 mM $Mg(OAc)_2$ (Baumann P and West SC, 1998b). That the magnesium concentration might be the critical difference is suggested by the findings of Wang H *et al.* They showed that their *in-vitro* end-joining reaction was dependent on DNA ligase IV only when performed in the presence of low (0.5 mM) Mg concentrations. Pre-incubation of cell free extracts with antibodies to DNA ligase IV had no inhibitory effect on end-joining when carried out at 10 mM Mg and the conclusion was that the contribution of DNA ligase IV to overall *in-vitro* end-joining was small (Wang H *et al.,* 2001b). More recently, Huang and Dynan describe an *in-vitro* DNA ligase IV/XRCC4 dependent DNA end-joining system that was carried out at an Mg concentration of 1.0 mM (Huang J and Dynan WS, 2002. We would need to repeat our assay using Manley extracts at low Mg concentrations to test for dependence on NHEJ factors.

In summary, 2 of our 5 cell lines derived from patients with late radionecrosis, LB0003 and LB0004 had abnormalities in DNA DSB repair. They demonstrated reduced DNA DSB rejoining associated with reduced in-vitro DNA-PK activity. All three subunits of DNA-PK were expressed at normal levels when examined by immunoblotting. The possible molecular defect(s) and mechanisms of these findings as well as future investigations are discussed in 7.1.

CHAPTER 6

RESULTS:

DNA DAMAGE SIGNAL TRANSDUCTION

IN THE NEWLY-DERIVED CELL LINES

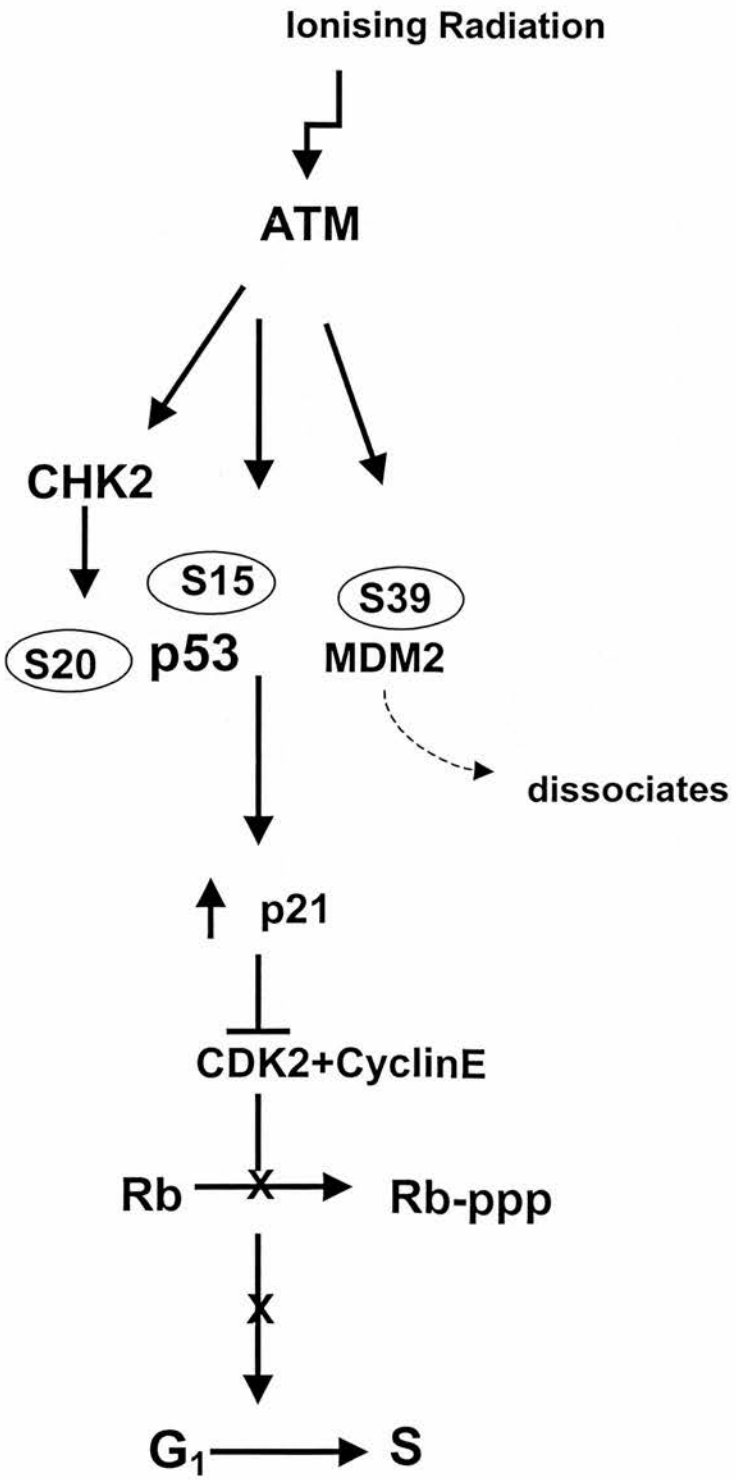
The role of DNA damage signal transduction in the radiation hypersensitivity of mammalian cells has been best studied in the human radiosensitivity syndromes, ataxia Telangiectasia (AT) and the Nijmegen Breakage Syndrome (NBS) [see sections 1.7b and 1.7c respectively]. In normal cells, the ATM (AT mutated) protein is rapidly activated by ionising radiation and radiomimetic agents leading to p53 phosphorylation at serine 15 and 20 (Siliciano JD *et al.,* 1997; Banin S *et al.,* 1998; Canman CE *et al.,* 1998; Khanna, KK, *et al.,* 1998). The phosphorylation of p53 inhibits the binding of MDM2 with p53 and protect p53 from ubiquitination and degradation (Shieh SY *et al.,* 1997). By promoting the stability of and thus activating p53 in response to ionising radiation, ATM induces a G1/S arrest. The retinoblastoma protein (pRB) functions downstream of p53 in the regulation of G1/S arrest following DNA damage. Following p53 activation, cyclin dependent kinases (cyclinA/CDK2, cyclinE/CDK2 and cyclinD1/CDK4) are inhibited leading to a loss of phosphrylation of pRB, which results in the cell cycle arresting at the G1/S checkpoint (Dulic V *et al.,* 1994; el-Deiry WS *et al.,* 1994). (Figure 6.1).

Studies attempting to link AT heterozygotes with the development of late radiation morbidity have given conflicting results (see section 1.3 and table 1.1). Nonetheless we hypothesised that one or more of our patients with late radionecrosis, might be heterozygous for an AT mutation. The biochemical components of the targets of ATM in the intra-S and G2/M checkpoints were less well characterised at the time of this study. Thus to study the functionality of the ATM signalling pathway in LB0002-6, we studied the proteins and processes involved in the ATM dependent G1/S DNA

Figure 6.1

The ATM dependent DNA damage G1/S checkpoint

(Modified from Shiloh Y, 2001)


Ionising Radiation
ATM
CHK2
S15
S20
p53
S39
MDM2
dissociates
p21
CDK2+CyclinE
Rb
Rb-ppp
G1
S

damage signal transduction pathway (sections 6.2 and 6.3). This was in addition to examining the cell lines for presence of the ATM protein (section 6.1).

Also at the time of this study, NBS1, mutations of which cause the Nijmegen Breakage Syndrome, had just been cloned (Carney JP *et al.,* 1998; Varon R *et al.,* 1998); the examination of the NBS1/Mre11/Rad 50 pathway was not included in this study.

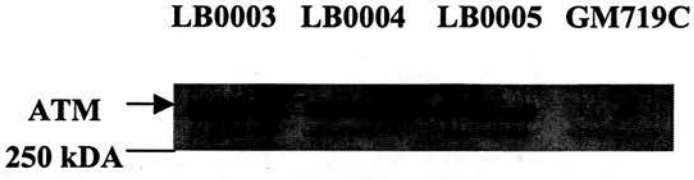
6.1 WESTERN BLOT ANALYSIS OF ATM

The majority of ATM mutations, in individuals with the radiosensitive syndrome ataxia-telangiectasia, are truncating mutations resulting in an unstable and undetectable gene product (Gilad S *et al.,* 1996; Telatar M *et al.,* 1996; Lakin ND *et al.* 1996). We thus hypothesised that one or more of the newly derived cell lines LB0002-LB0006 might be heterozygous for a truncating mutation in ATM. We sought to examine this using western blot analysis. Cell line extracts were accordingly probed for the presence of the 350 kDa ATM protein. ATM was shown to be present in all the cell lines at the correct molecular weight. Therefore none of the cell lines had a truncation mutation in ATM. This was not unexpected for LB0006 given that the in-vitro radiation sensitivity of the cell line was normal. In particular, LB0005 (see sections 6.2 and 6.3 below) as well as the cell lines (LB0003 and LB0004) with reduced DNA-PK activity (see section 5.2) had normal ATM levels (Figure 6.2). The latter is in contrast to the glioma cell line M059J which lacks both DNA-PKcs and ATM expression (Chan DW, *et al.,* 1998).

Figure 6.2

Western blotting for the presence of ATM

50 μg of whole cell extract from an AT cell line, GM719C, and newly derived cell lines LB0003, LB0004 and LB0005 were loaded onto a 6% SDS-polyacrylamide gel (ratio of acrylamide to bisacrylamide = 100:1). Following separation by electrophoresis, the proteins were transferred onto nitrocellulose membrane and then probed with an antibody to ATM. All the newly derived cell lines showed equal amounts of ATM as exemplified by the 3 cell lines shown.


LB0003 LB0004 LB0005 GM719C
ATM
250 kDA

6.2 POST-IRRADIATION P53 UP-REGULATION BY WESTERN BLOT ANALYSIS

In spite of expressing normal amounts of full length ATM protein, we hypothesised that the newly-derived cell lines might have decreased ATM activity due to a missense mutation (Stankovic T *et al.,* 1998). Cell lines from AT individuals have reduced and delayed p53 up-regulation following ionising radiation (Kastan MB *et al.,* 1992; Khanna KK and Lavin MF, 1993). To confirm that ATM activity was normal in the newly derived cell lines, the up-regulation of p53, a target of ATM following DNA damage, was measured after 2 or 4 Gy ionising radiation. Unsynchronised cells were irradiated and returned to the incubator at 37^0C, 5% CO_2. Aliqouts of cells were harvested at 0.5, 1, 2 and 4h post-irradiation and analysed for p53 expression by western blot analysis as described in section 3.15.

Initially p53 was present in all the unirradiated cell lines (data not shown). By 2 hours post-irradiation with 4 Gy, a 2 to 3 fold up-regulation of p53 was observed in the newly derived cell lines, except for **LB0005** (Figure 6.3). This degree of p53 up-regulation is consistent with previous reports for normal lymphoblastoid cell lines (Khanna KK and Lavin MF, 1993; Mirzayans R *et al.,* 1995) indicating normal ATM activity in the cell lines apart from LB0005. In particular, both LB0003 and LB0004, the two cell lines with reduced DNA-PK activity showed normal kinetics and levels of p53 up-regulation. This would be in agreement with the observation that p53 up-regulation is normal in cells with deficient DNA-PK activity (Burma S *et al.,* 1999; Jimenez GS *et al.*, 1999; Jhappan C *et al.,* 2000).

Figure 6.3

p53 upregulation by 4 Gy ionising radiation

The relative amounts of p53 protein in extracts of the indicated cell lines prepared at 2 and 4 h after exposure to 4 Gy ionising radiation as described in legend of figure 6.2. SNC 4 is a normal cell line and GM719C is that from an individual with ataxia telangiectasia. The bars show means from at least 3 experiments, error bars show mean +/- 1.0 SE.

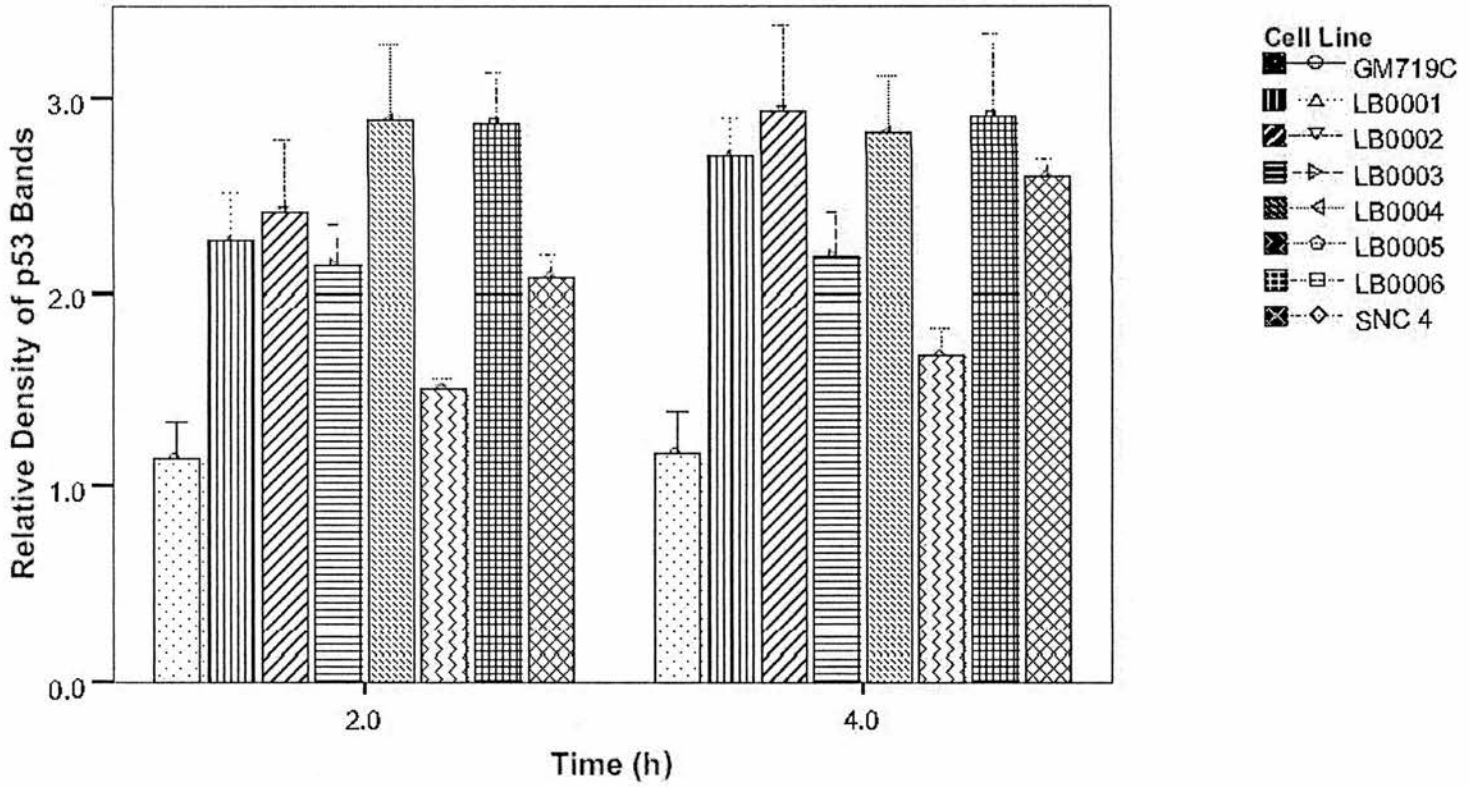
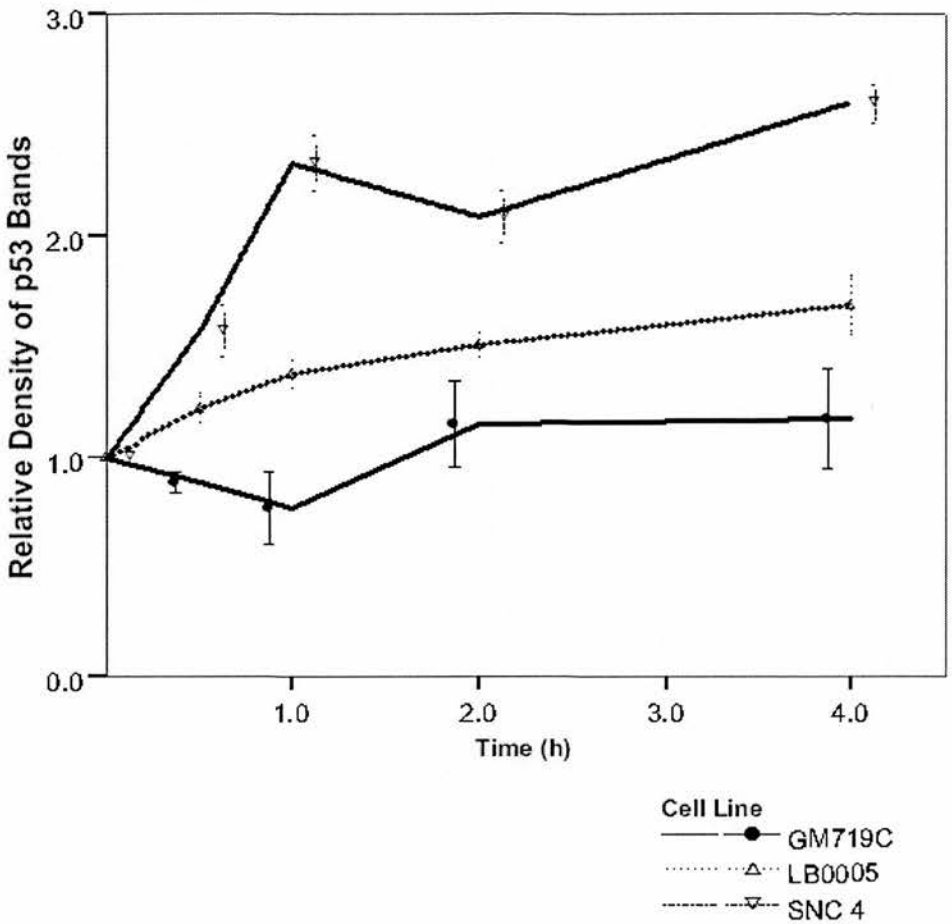

Relative Density of p53 Bands
3.0
2.0
1.0
0.0
2.0
4.0
Time (h)
Cell Line
GM719C
LB0001
LB0002
LB0003
LB0004
LB0005
LB0006
SNC 4

Figure 6.4 and Table 6.1

p53 up-regulation in LB0005

The time-course of p53 up-regulation by 4 Gy of ionising radiation for LB0005, is compared graphically (Figure 6.4) and in a table (Table 6.1) with a normal cell line SNC 4 and GM719C, a cell line from an individual with ataxia telangiectasia. The means are from at least 3 experiments. The error bars (Figure 6.4) show mean +/- 1 SE. The p values (Table 6.1) are for the comparison of the means between SNC 4 and LB0005 at each of the time-points.



Time post-irradiation	Mean Relative Density of p53 bands			
	SNC 4	**LB0005**	**GM719C**	**p value** (comparing SNC 4 versus LB0005)
Half h	1.58	1.23	0.89	p=0.03
1h	2.33	1.38	0.77	p=0.001
2h	2.09	1.51	1.15	p=0.004
4h	2.60	1.69	1.17	p=0.001

Although LB0005 also demonstrated up-regulation of p53 in response to ionising radiation, it had significantly reduced levels of p53 post-irradiation with 4 Gy at each of the time-points examined (Figure 6.4). The levels of p53 up-regulation at each time-point were intermediate between that of the normal control cell line and that of an AT cell line GM719C (Table 6.1). These data need further investigation to determine whether cell line LB0005 carries a heterozygous missense mutation in or a polymorphic variant sequence of ATM (see section 7.2).

6.3 POST-IRRADIATION RETINOBLATOMA PROTEIN (pRB) PHOSPHORYLATION

pRb and Rb-related proteins have been shown to be checkpoint participants downstream of p53 in the p53–mediated G1 arrest in response to DNA damage. (Dulic V *et al.,* 1994; el-Deiry WS *et al.,* 1994; Slebos RJC *et al*, 1994).

Khanna KK *et al* found in unirradiated control cells, the ratio of hyper-phosphorylated/hypo-phosphorylated pRb was approximately one and that following exposure to ionising radiation, there was an accumulation of the hypo-phosphorylated (more rapidly migrating) form of pRb. In unirradiated AT cells, the ratio of hyper-phosphorylated-to-hypo-phosphorylated Rb was approximately 5:1. Moreover, there was either only a small or no increase in the level of hypo-phosphorylated pRb at 2h and 12h post-irradiation with 3 Gy (Khanna KK *et al.,* 1995).

To confirm that normal ATM activity and p53 induction were associated with an expected accumulation of hypo-phosphorylated pRb in the newly derived cell lines,

immunoblots of cell lysates were performed at 4 and 20h after exposure to 4 Gy. This was compared against AT cell line BD 2630. The data was very much in accordance with the findings of Khanna *et al.* In unirradiated BD2630, nearly all the pRb was in the hyper-phosphorylated form and there was virtually no conversion to the hypo-phosphorylated form upon irradiation (Figure 6.5).

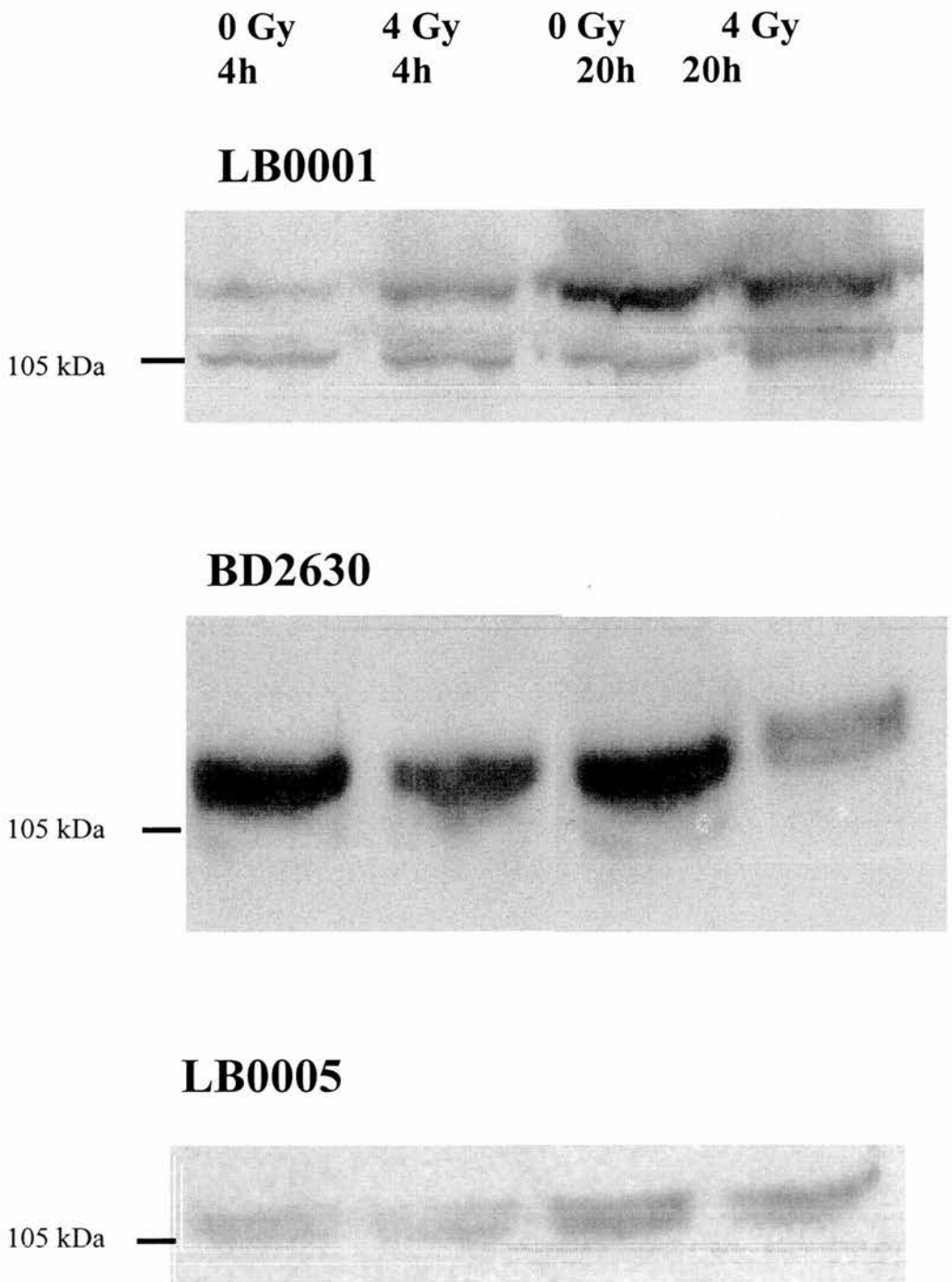
LB0005 did show accumulation of hypo-phosphorylated pRb after exposure to ionising radiation but the degree of accumulation was reduced compared to LB0001 as assessed qualitatively (Fig 6.4). In all the other newly derived cell lines, namely LB0002, LB0003, LB0004 and LB0006, there was an accumulation of the hypophosphorylated pRb upon exposure to ionising radiation (data not shown). Under the experimental conditions described, resolution of the two bands representing hyper- and hypo-phosphorylated pRb was not sufficient to allow densitometric quantification and comparison between the cell lines.

In summary, examination of components of the ATM dependent G1/S pathway in the cell lines LB0002-6 from individuals who developed severe late radiation injury revealed abnormality in only one of the cell lines, namely, LB0005. LB0005 demonstrated reduced levels of p53 up-regulation in response to ioinising radiation (Fig 6.3 and 6.4) even though it expressed normal levels of ATM of the expected molecular weight (Fig 6.1). In addition conversion to hypo-phosphorylated pRb occurs in response to ionising radiation, implying that G1/S is not absent. Further elucidation of the G1/S and indeed the G2/M and intra-S radiation response in LB0005 is required and future experiments are discussed in chapter 7.

Figure 6.5

Phosphorylation status of pRb after exposure to ionising radiation determined by immunoblotting.

Unsynchronized cells were exposed to 4 Gy. 100 μg of whole cell extracts were loaded onto a 6% SDS-polyacrylamide gel and the proteins separated by electrophoresis. The proteins were transferred onto nitrocellulose membrane and probed with antibody to retinoblastoma protein. The more rapidly migrating band is the hypophosphorylated form of pRb. Its presence in the irradiated extracts indicates intact G1/s checkpoint following exposure to ionising radiation. Conversion to hypophosphorylated pRb was detected 4h and 20h after exposure to ionizing radiation in LB0001 and all the other newly derived cell lines, including LB0005 but not in BD 2630, an AT cell line.


0 Gy 4h
4 Gy 4h
0 Gy 20h
4 Gy 20h
LB0001
105 kDa
BD2630
105 kDa
LB0005
105 kDa

CHAPTER 7

DISCUSSION AND CONCLUSION

DISCUSSION

Severe late radiation injury, either necrosis or fibrosis, is dose-limiting for curative radiotherapy, and occurs in about 5% of non-syndromic patients receiving this treatment. It has been suggested that these patients may be intrinsically more radiosensitive, although they appear to form a continuum with the remaining 95% of the normal population (Busch D, 1994; Burnet NG *et al.,* 1992) and that identifying these inherently sensitive individuals will allow the delivery of a higher radiation dose to other patients, in turn improving their chance of cure (Tucker SL *et al.,* 1996). Prospective studies of intrinsic radiosensitivity using clonogenic assays have shown a correlation of the former with patient survival in patients with cervix (West CM *et al.,* 1997) and head and neck cancer (Bjork-Eriksson T *et al.,* 1998) and with late tissue morbidity in patients with breast (Loeffler JS *et al.,* 1990), cervix (West CML *et al.,* 2001) and other cancers (Alsbeih G *et al.,* 2000). However 2 large studies (Russell NS *et al.,*1998, Peacock J *et al.,* 2000) have suggested that the relationship between intrinsic radiosensitivity and late tissue morbidity is not strong enough for accurate prediction (see also Chapter 1.3b). Possible reasons for this discrepancy have been discussed in chapter 1.3b. Thus a different approach is required and this might include the identification of genetic or biochemical defects that predispose individuals to severe normal tissue morbidity.

Two reports have suggested that **DNA repair proficiency** might be important. Herring and coworkers found a correlation between expression of the human AP endonuclease HAP1, determined immunohistochemically in formalin-fixed paraffin-embedded tissue, and clonogenic survival after clinically relevant doses of radiation in the patients with cervix cancer described above (Herring CJ *et*

al., 1998). Alapetite and colleagues used the alkaline comet assay to investigate strand-break rejoining in lymphocytes from 26 patients with severe radiation reactions, 22 with normal reactions and 24 healthy donors. All 6 patients with the most severe RTOG grade 4 reactions exhibited impaired strand-break rejoining (Alapetite C *et al.,* 1999). The controls included patients who had been irradiated several years before, suggesting that the impaired rejoining was not a direct consequence of the radiotherapy. Severin DM *et al* sequenced the *hHR21*(human rad21) gene in 19 radiation-sensitive cancer patients. Rad21 is involved in the repair of DNA DSBs in Schizosaccharomyces pombe. In 6 of the 19 patients, a thymidine to cytosine transition, which does not result in change in amino acid, was detected at position 1440 of the *hHR21* open reading frame (T1440C). One patient with a particularly severe radiation reaction had a second sequence variant immediately adjacent to the first. This was a guanine (G) to adenine (A) transition (G1441A), resulting in a change of the amino acid sequence (glycine --> arginine) in a portion of the protein conserved in evolution Severin DM *et al.,* 2001). More recently, Andreassen CN *et al* examined and found positive correlation between clinical normal tissue radiosensitivity and single nucleotide polymorphisms in 5 candidate genes including *XRCC3*, a gene important in homologous recombination.

Taken together, these studies strongly suggest that DNA repair activity might influence radiosensitivity in the clinical setting. The predominant lesion responsible for lethality is the DSB, and the major repair pathway for its correction in mammalian cells is non-homologous end-joining (NHEJ). Hence one of our hypotheses was that abnormalities in DNA repair, particularly in the NHEJ pathway might be associated with the development of severe, late radiation morbidity. At the

time we began the study, ataxia-telangiectasia was the only human radiosensitive syndrome that had been well characterised biochemically and accordingly we also investigated the G1/S DNA damage signalling pathway orchestrated by the ATM protein.

Before performing the in-vitro work, we first retrospectively analysed a defined cohort of patients treated at the Western General Hospital in Edinburgh. These were patients who had received radical radiotherapy for carcinoma of the cervix between 1974 and 1988. The main aim of the study was to decide if a radiation-sensitive subgroup, similar to that published in the literature, could be identified in the South East Scottish population. Using a cut-off dose of 75Gy, which most centres would consider safe, we found that about 4% of patients developed a grade 4 or 5 RTOG late morbidity. Hence it was likely that a radio-sensitive subpopulation as described in the literature was also present amongst South East Scottish cancer patients.

We then proceeded to identify these radiation-sensitive patients from a departmental database, which had been established in 1974 and now contains data of over 70 000 patients. Our first eligibility criteria were patients who had developed RTOG 4 (appendix) late radiation injury following treatment with standard radiotherapy doses. We hypothesised that patients developing late injury as a result of an underlying genetic abnormality in, for example, DNA repair might also be cancer-prone, and accordingly selected patients under the age of 50 years for this study. We also decided in the first instance to shortlist patients with either breast or cervix cancers. Breast cancer patients were selected because of the association of breast cancer with carriers of the AT gene (Khanna JNCI, etc). The *BRCA1* and

BRCA2 genes were also found to be involved in homologous recombination (Scully R *et al.*, 1997; Sharan SK *et al.*, 1997). More recently, large genetic association studies have implicated implicated rare polymorphic variants of two *Rad51* paralogs, namely, *XRCC2 (R188H)* and *XRCC3* in breast cancer susceptibility especially in younger patients with a positive family history (Rafii S *et al.,* 2002; Kuschel K *et al.,* 2002). This further supported our hypothesis that patients with severe radiation injury could have a genetic defect that predisposed them to both early onset breast cancer as well as the former.

Radiotherapy (with chemotherapy) is still the radical treatment modality for advanced staged cervical cancer. The radiotherapy dose is limited by the tolerance of surrounding normal structures especially the small bowel and rectal wall. By identifying patients who are predisposed to radiation injury, it is possible that doses for the remaining patients with cervical cancer can be escalated, thereby improving the local control and cure rates. Thus we studied patients with cervical cancer and late radiation injury to try and identify biochemical abnormalities that might be developed as predictive assays (see chapter 1.2 and 1.3b). Patients with necrosis rather than fibrosis were selected because we thought a cell-death endpoint was more likely to be related to a DNA repair defect. Indeed the severe, sometimes fatal radiation morbidity, in patients with ataxia-telangiectasia and in the patient from whom the DNA ligase IV defective cell line 180BR was derived, is likely to be a result of cell death (Gotoff SP *et al.*, 1967; Morgan JL *et a.,* 1968; McFarlin DE *et al.,* 1972; Plowman PN *et al.,*1990).

Having identified the patients, we proceeded to establish Epstein-Barr immortalised lymphoblastoid cell lines from 5 patients that met the criteria described above (LB0002-6) and 1 patient that had an increased acute skin erythema which resolved over time and in whom no late damage has occurred (LB0001). The decision to establish lymphoblast as opposed to fibroblast cell lines was based on several factors. Two important considerations were firstly, the efficiency of immortalisation and subsequent growth to produce sufficient cell numbers for biochemical assays using whole cell extracts. For this study all 6 lymphocyte samples were successfully immortalised. The second important factor was patient acceptability of venepuncture in order to obtain the lymphocytes for immortalisation. Moreover EBV-immortalised cell lines are polyclonal and therefore should be representative of the B cells of the patients with respect to radiation sensitivity, genetic and biochemical studies. The main disadvantage of using lymphoblastoid cell lines is that standard clonogenic assays, the gold standard by which *in-vitro* radiation sensitivity is measured, could not be performed. Consequently, there is far less published data to compare, for example, the SF2 (surviving fraction at 2 Gy) of these newly established cell lines than if they had been fibroblast cell lines.

After the cell lines were established, we examined the karyotype of the cell lines by giemsa staining of metaphase spreads, for evidence of spontaneous chromosomal aberrations which would indicate an underlying chromosomal instability syndrome, and found LB0001-LB0006 all to be 46 XX.

A summary of the main in-vitro findings for each of the cell lines is presented in the following.

7.1 LB0003 and LB0004

LB0003 was derived from an individual with early onset breast cancer at age 40 years which was treated by mastectomy and adjuvant radiotherapy: 45 Gy in 10 fractions over 28 days. She developed chest wall necrosis requiring chest wall reconstruction 17 years later, a contralateral primary breast cancer after 18 years and a presumed ipsilateral lung cancer (from which no confirmatory histology was obtained) 26 years later, perhaps indicating a cancer-proneness. The cell line showed in-vitro sensitivity to radiation with an SF_2 of 0.2097. PFGE data for the cell line demonstrated no difference in the amount of initial DNA DSBs induced by a given dose of ionising radiation. Instead there was a significant reduction in the proportion of DSB repaired by the fast kinetic pathway. For example, at half hour post-irradiation with 30 Gy, just 5% of the initial amount of DNA DSBs had been rejoined (compared to 60% for the control cell lines).

Cell line **LB0004** was derived from a patient who had a FIGO stage IB primary squamous cell carcinoma of the cervix diagnosed at age 43 years. Two years following a conventional protocol of radiotherapy, she developed vesico-vaginal and ileo-vesical fistulae. The in-vitro findings are similar to LB0003. The cell line showed in-vitro sensitivity to radiation with an SF_2 of 0.1992. There was a significant reduction in the proportion of DSB repaired by the fast kinetic pathway. Like LB0003, at half hour post-irradiation with 30 Gy, just 5% of the initial amount of DNA DSBs had been rejoined (compared to 60% for the control cell lines).

Both these cell lines had significantly reduced DNA-PK activity as measured in-vitro: LB0003 by 2.5-8 fold and LB0004 by 3-10 fold compared to control cell lines. In both LB0003 and LB0004, the expression of all three components of the

DNA-PK heterotrimer as well as of XRCC4 and DNA ligase IV, by western blot, was normal. The G1/S response to ionising radiation as measured by p53 up-regulation was intact and both cell lines expressed ATM.

Other groups have examined the correlation between DNA-PK activity and radiosensitivity using human material. There is conflicting evidence utilising tumour cell lines. Three groups have found a very strong correlation between double-strand break rejoining, radiosensitivity and DNA-PK activity in 5 lung cancer cell lines (Sirzen F *et al.,* 1999) and in 2 squamous cell cancer cell lines (Polischouk AG *et al.,* 1999), and between radiosensitivity and DNA-PK activity in 14 oesophageal cancer cell lines (Zhao HJ *et al.,* 2000). In contrast one group reported no correlation between DNA-PK activity and radiosensitivity in 9 glioma cell lines (Allalunis-Turner J *et al.,* 1995). This may indicate that other factors are more important in determining radiosensitivity in gliomas, which clinically are highly radioresistant. Decreased DNA-PK activity was also observed in 6 human cancer cell lines exhibiting low-dose radiation hypersensitivity (Vaganay-Juery S *et al.,* 2000). Two studies could not relate DNA-PK activity to radiosensitivity when this was assayed in fibroblasts (Kasten U *et al.,* 1999; Carlomagno F *et al.,* 1999). In aggregate, these various studies suggest that DNA-PK activity may be an important factor affecting radiosensitivity in humans, but with cell type-specific variation.

One group has examined NHEJ enzyme expression, as well as activity, in relation to late radiation injury (Carlomagno F *et al.,* 1999). They reported that there was no correlation between expression and activity of the NHEJ enzymes DNA-PKcs, Ku70, Ku80, XRCC4 and DNA ligase IV, and the homologous recombination enzyme rad51 and the risk of developing severe telangiectasia. An early study in this

field reported clinical evidence that there were different determinants of specific biological endpoints (Bentzen SM *et al.,* 1993). The absence of a relationship between DNA repair enzyme activity and clinical response in this series may have reflected the choice of a clinical endpoint to which these activities did not contribute, and may not apply to other, particularly necrosis-related, endpoints.

The reduced DNA-PK activity we have observed in the 2 EBV-immortalised cell lines does not seem to be an artefact of transformation or of previous radiotherapy. Normal levels of DNA-PK activity were seen in the 2 control EBV-transformed cell lines (Macsi and Wales) and the other newly derived cell lines (LB0001, LB0002, LB0005 and LB0005) we have examined. Four of these were derived from patients who had received radiotherapy previously.

No humans with a DNA-PKcs or Ku null phenotype have been described to date. While the two cell lines described here exhibit substantially reduced DNA-PK activity, both do have some residual function and express all 3 protein components of DNA-PK. It is possible that this reduced level of activity is sufficient for function under normal conditions, but insufficient to deal with the extent of DNA repair required following radiotherapy. Indeed while transgenic mice null for DNA-PKcs are viable, animal models have suggested that with increasing size of the mammalian species, the expression of DNA-PK activity in normal fibroblasts increases and the SCID phenotype in affected animals becomes correspondingly more severe (Meek K *et al.,* 2001). It is possible that a germ-line DNA-PKcs null phenotype is not compatible with viability in humans.

Our limited, preliminary sequencing analysis (Chapter 5.2) did not reveal any sequence variation from published databases but we cannot on the current evidence

exclude mutations or polymorphisms within the genes encoding one of the 3 DNA-PK subunits, affecting either protein-protein interactions, DNA-binding or the kinase activity. Indeed, polymorphisms in certain DNA repair genes have been found to correlate with the risk of severe normal tissue morbidity (Severin DM *et al.,* 2001; Andreassen CN *et al.,* 2003). Future work would involve, in the first instance, sequencing to exclude missense mutation of polymorphisms in the *DNA-PKcs* gene.

7.2 LB0005

Cell line **LB0005** was derived from a patient who had a FIGO stage IIIB primary squamous cell carcinoma of the cervix diagnosed at age 40 years. Two years following a conventional protocol of radiotherapy, she developed a recto-vaginal fistula. We were unsuccessful with obtaining in-vitro radiation sensitivity data for LB0005 using our proliferation assay. The assay was performed in 96 well plates at 4-5 days post-irradiation to allow for recovery of exponential growth in the irradiated cell populations; after irradiation, LB0005 cells were not able to survive in culture in the 96 well plates for that period of time. We plan to use an assay for apoptosis to test in-vitro sensitivity of LB0005 to ionising radiation. Apoptosis in lymphoblastoid cell lines has been measured at 24-48h (Takagi M *et al.,* 1998; Crompton NEA *et al.,*1999) post-irradiation and LB0005 should be able to survive for this period in culture.

The in-vitro DNA-PK activity in LB0005 was not significantly different from that in normal controls and there was normal expression of all three sub-units of DNA-PK and of XRCC4 and DNA ligase IV.

LB0005 was the only cell line in the study to demonstrate reduced p53 up-regulation in response to ionising radiation. It also expressed normal levels of ATM of the expected weight and a degree of conversion to hypophosphorylated pRb after ionising radiation exposure indicating that G1/S arrest post-radiation is not absent. Cell cycle progression following ionising radiation needs to be examined using flow cytometry to confirm an abnormal G1/S response that is suggested by the reduced p53 up-regulation and to quantify the fraction of cells that do arrest at G1. In addition G2/M and S-phase checkpoints post-irradiation need to be examined both using flow cytometry and by immunoblotting for phospho-proteins that are phosphorylated in an ATM dependent manner. These proteins would include CDC25C which is involved in the G2/M checkpoint and NBS1 which is involved in the intra-S checkpoint (Shiloh Y, 2003).

Certainly if all three checkpoints are found to be compromised in response to ionising radiation, then mutations or polymorphisms in ATM that are associated with production of full length message and stable protein could still be the underlying abnormality in LB0005. Sequencing of ATM gene would then be required. Angele *et al* reported that LCLs, from radiosensitive breast cancer patients, containing ATM polymorphism exhibited a lower p53 induction when compared with normal controls (Angele S *et al.,* 2003). Similarly Takagi *et al* found that ATM-dependent DNA damage responses, including serine 15 phosphorylation of p53, was abnormal in LCLs with single nucleotide variant ATM from paediatric patients with Hodgkin Disease (Takagi M *et al.,* 2004).

Alternative molecular defect/s could also account for the abnormality in LB0005 especially if the flow cytometry and other experiments discussed above

reveal that the defect involves the G1/S checkpoint alone. For example, Dikomey *et al* recently described a molecular mechanism of radiosensitivity in human fibroblasts that impaired both DNA DSB rejoining and G1/S arrest following ionising radiation (Dikomey E *et al.,* 2003b). This was not due to defects in DNA-PK and they have yet to pin down the underlying genetic or biochemical abnormality; the same abnormality may underlie the defect in LB0005.

7.3 LB0002 and LB0006

LB0002 was derived from a patient who presented with early onset breast cancer at age 38 years and was treated by lumpectomy and adjuvant radiotherapy without systemic chemotherapy. She received 40 Gy in 10 fractions over 25 days were prescribed using external beam treatment followed by 42 Gy delivered by low dose-rate (0.46 Gy per hour) brachytherapy. She developed skin necrosis 14 years later. Although this may have arisen from the large doses delivered, the cell line did demonstrate in-vitro sensitivity to radiation that was less pronounced than that of LB0003 and LB0004 with an SF_2 of 0.2758. Apart from an increased in-vitro radiation sensitivity, there were no abnormal findings in this study. DNA-PK activity was not significantly different from the control cell lines and the expression of Ku70, Ku80, DNA-PKcs, XRCC4 and DNA ligase IV were all normal. Future work for this cell line could include testing for DNA ligase IV/XRCC4 activity using an adenylation assay (Riballo E *et al.,* 1999).

ATM expression and p53 up-regulation following ionizing radiation were both normal. Cell cycle progression following ionising radiation by flow cytometry especially for the G2/M and S-phase blocks need to be investigated since this study only focussed on the ATM dependent G1/S arrest. Finally another

potential plan of investigation could be into the role played by both BRCA1, BRCA2 and rare variants of *XRCC2* and *XRCC3*in this patient with early onset breast cancer (Rafii S *et al.,* 2002; Kuschel K *et al.,* 2002).

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LB0006 was derived from a patient who presented with early onset breast cancer at age 38 years and was treated by mastectomy and adjuvant radiotherapy (without systemic chemotherapy), receiving 44 Gy in 10 fractions over 26 days. She developed necrosis of the ipsilateral 4^{th} and 5^{th} ribs 2 years later. The cell line displayed the same level of cellular sensitivity as the controls with an SF2 of 0.3787. One reason for this is that factors other than cellular sensitivity may account for the rib necrosis sustained by the patient (Marcou Y *et al.,* 2001). No abnormal in-vitro findings were found in LB0006 in this study.

Previous reports of increased clinical radiosensitivity in AT, ATLD, NBS and 180BR (Plowman PN *et al.,* Badie C *et al.,* 1995) have been associated with extreme, and often fatal, radiosensitivity, and in the first 3 with very rare syndromes with underlying neurological and immunological abnormalities (Gotoff SP *et al.,* 1967; Taalman RD *et al.,* 1983; Stewart GS *et al.,* 1999). The patients from whom we have derived these cell lines were clinically normal before radiotherapy, in particular had no evidence of neurological or immunological abnormality, other than to have developed a cancer at a relatively early age. They have exhibited comparatively milder degree of increased radiosensitivity, with radiation injury occurring many years after treatment, rather than requiring its cessation.

As the incidence of cancer continues to rise, the use of ionising radiation will continue to increase and so to the need to try and identify patients who are at risk of

radiation injury. In contra-distinction to the single gene disorders that cause the clinical syndromes described above, it has been postulated that these more typical cases of increased clinical radiosensitivity arising in non-syndromic cancer patients may be a complex trait (Andreassen CN *et al.,* 2002). If so, then future predictive testing may include a battery of tests rather than a single one. In this study, 3 of 5 cell lines established from patients with late radionecrosis revealed biochemical abnormalities. Although the precise biochemical abnormality has not been determined for any of the cell lines in this study, continued investigation should add to an understanding of the molecular mechanisms underlying normal tissue radiosensitivity. There should be continued establishment and investigation of more cell lines along the lines pursued in this work. Then taken with work performed in other laboratories and in collaborations such as the GENEPI project by ESTRO, a battery of tests may yet be developed for predictive testing.

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APPENDIX

RTOG LATE RADIATION MORBIDITY SCORING SCHEMA*

(www.**rtog**.org/members/toxicity/**late**.html)

	0	1	2	3	4
Small/large intestine	None	Mild diarrhea Mild cramping Bowel movement 5 times daily Slight rectal discharge or bleeding	Moderate diarrhea and colic Bowel movement >5 times daily Excessive rectal mucus or intermittent bleeding	Obstruction or bleeding requiring surgery	Necrosis/ Perforation Fistula
Bladder	None	Slight epithelial atrophy Minor telangiectasia (microscopic hematuria)	Moderate frequency Generalized telangiectasia Intermittent macroscopic hematuria	Severe frequency and dysuria Severe generalized telangiectasia (often with petechiae) Frequent hematuria Reduction in bladder capacity (<150 cc)	Necrosis/ Contracted bladder (capacity <100 cc) Severe hemorrhagic cystitis
Bone	None	Asymptomatic No growth retardation Reduced bone density	Moderate pain or tenderness Growth retardation Irregular bone sclerosis	Severe pain or tenderness Complete arrest of bone growth Dense bone sclerosis	Necrosis/ Spontaneous fracture
Skin	None	Slight atrophy Pigmentation change Some hair loss	Patchy atrophy; Moderate telangiectasia; total hair loss	Marked atrophy; Gross telangiectasia	Ulceratiom
Subcutaneous tissue	None	Slight induration (fibrosis) and loss of subcutaneous fat	Moderate fibrosis but symptomatic. Slight field contracture <10% linear reduction	Severe induration and loss of subcutaneous fat. Slight field contracture >10% linear measurement	Necrosis

	0	1	2	3	4
Lung	None	Aymptomatic or mild symptoms (dry cough). Slight radiographic appearances	Moderate symptomatic fibrosis or pneumonitis (severe cough). Low grade fever Patchy radiographic appearances	Severe symptomatic fibrosis or pneumonitis Dense radiographic appearances	Severe respiratory insufficiency/ Continuous O_2/Assisted ventilation
Heart	None	Aymptomatic or mild symptoms Transient T wave inversion & ST changes Sinus tachycardia > 110 (at rest)	Moderate angina on effort Mils pericarditis Normal heart size Persistent abnormal T wave amd ST changes Low QRS	Severe angina Pericardial effusion Constrictive pericarditis Moderate heart failure Cardiac enlargement ECG abnormalities	Tamponade/Sev ere heart failure/Severe constrictive pericarditis

*as applicable to breast and pelvic irradiation.

Note: RTOG grade 5 morbidity is defined as death directly related to radiation late effects

There is no RTOG grading for late morbidity of the vagina and in this site, grade 4 morbidity was defined as vaginal vault necrosis.

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Reduced DNA-dependent protein kinase activity in two cell lines derived from adult cancer patients with late radionecrosis

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Epstein–Barr virus-immortalized lymphoblastoid cell lines were derived from five patients with late radionecrosis. Two of these cell lines exhibited postradiation viability levels intermediate between normal cell lines and that from an individual with ataxia telangiectasia. Compared with controls, these two cell lines exhibited impaired ability to rejoin DNA double-strand breaks on pulsed-field gel electrophoresis and 6–10-fold reduced DNA-dependent protein kinase (DNA-PK) activity *in vitro* in cell-free extracts. Immunoblotting showed normal levels of Ku70, Ku80 and XRCC4 and the presence of DNA-PKcs in both cell lines. These findings suggest that DNA-PK might be an important factor affecting the predisposition of radiotherapy patients to late radionecrosis.
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The cancerocidal effect of radiotherapy is dependent on delivered dose. This is limited by normal tissue tolerance. However, at radiation dose-schedules well tolerated by most individuals, some patients develop late radionecrosis. The factors accounting for these inter-individual differences are unknown (Safwat *et al.*, 2002).

Four human radiosensitivity syndromes are related to defective DNA repair: ataxia telangiectasia (AT) (Taylor *et al.*, 1975), Nijmegen breakage syndrome (NBS) (Taalman *et al.*, 1983), ataxia telangiectasia-like disease (ATLD) (Stewart *et al.*, 1999) and DNA ligase IV deficiency (O'Driscoll *et al.*, 2001). All are rare autosomal recessive conditions. Affected individuals have often been children with severe, sometimes fatal, toxicity from conventional radiotherapy.

Most patients with late radionecrosis are adults with no preradiation phenotype. They may be intrinsically more radiosensitive, but form a continuum with the 'normal' population. Identifying these sensitive individuals may allow a higher radiation dose to be delivered to other patients, with improved chance of cure (Tucker *et al.*, 1996). Studies of intrinsic radiosensitivity have shown a correlation with patient survival (West *et al.*, 1997; Bjork-Eriksson *et al.*, 1998) and late tissue morbidity (West *et al.*, 1998; Alsbeih *et al.*, 2000). They have not identified the underlying biochemical mechanisms. Two reports have suggested the importance of DNA repair proficiency. Herring *et al.* (1998) found a correlation between HAP1 expression and clonogenic survival after clinically relevant doses of radiotherapy. Alapetite *et al.* (1999) observed impaired strand-break rejoining in lymphocytes from six patients with RTOG grade 4 reactions. The control patients included individuals who had been irradiated several years previously, indicating the impaired rejoining was not consequent upon radiotherapy.

Taken together, these studies suggest that DNA repair activity affects clinical radiosensitivity. The predominant ionizing radiation-induced lethal DNA lesion is the double-strand break (DSB) (Iliakis, 1991). Its major repair pathway is nonhomologous end-joining (NHEJ). To explore the role of DNA repair in adult cancer patients with late radionecrosis, we derived cell lines from five patients with RTOG grade 4 late radionecrosis. We hypothesized that patients developing radionecrosis due to impaired DNA repair might be cancer-prone, and selected patients under age 50 years. Patients with necrosis but not fibrosis were selected because a cell-death end point was thought to be more likely related to impaired DNA repair than a proliferative end point such as fibrosis. Local Research Ethics Committee approval was obtained and patients provided written informed consent.

Initially, we investigated the expression and activity of enzymes involved in NHEJ in the five cell lines. We report two cell lines that exhibit reduced post-radiation viability, impaired DSB rejoining and reduced DNA-dependent protein kinase (DNA-PK) activity *in vitro*.

Cell line LB0003 was derived from a patient who presented with breast cancer at 40 years of age, treated by mastectomy and adjuvant radiotherapy. She developed chest wall necrosis requiring reconstruction after 17 years, contralateral primary breast cancer after 18 years and a presumed ipsilateral lung cancer 26 years later.

Cell line LB0004 was derived from a patient with FIGO stage Ib squamous cell cervix carcinoma at 43 years of age, treated with external beam and intracavi-

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Table 1 Characteristics of control cell lines

	Clinical characteristics	*Origin*	*Transformed at*
LB0001	Breast cancer patient, age 29 years; severe acute radiation reaction	Edinburgh Cancer Centre	ECACC, Salisbury, UK
Macsi	Patient with ocular abnormality; no cancer	Professor Veronica van Heynigen, MRC HGU	MRC Human Genetics Unit, Edinburgh, UK
Wales	Patient with ocular abnormality; no cancer	Professor Veronica van Heynigen, MRC HGU	MRC Human Genetics Unit, Edinburgh, UK
BD 2630	Individual with AT	ECACC, Salisbury, UK	ECACC, Salisbury, UK

tary radiotherapy. After 2 years, she developed vesicovaginal and ileovesical fistulae. Both fistulae were managed surgically and she remains disease-free 10 years later. Control cell lines used were all immortalized with Epstein–Barr virus (EBV) (Table 1).

LB0003 and LB0004 exhibit moderately increased radiosensitivity

To determine whether these patients were predisposed to radionecrosis by increased cellular radiosensitivity, we assayed postradiation viability in LB0003, LB0004, positive and negative control cell lines. We chose a viability assay because of the difficulty associated with colony-forming clonogenic survival assays with lymphoblastoid cells in suspension. The number of proliferating cells after 0, 0.5, 1.0 and 2.0 Gy was determined for the cell lines LB0003 and LB0004, derived from patients with late radionecrosis, and for LB0001, Macsi, Wales and BD 2630. The postradiation viability curves for LB0003 and LB0004 were intermediate between those of the normal controls and the acute over-reactor (LB0001), and the AT cell line (Figure 1). After 2 Gy, the proportion of cells remaining viable was 38.1% for Wales, 38.2% for Macsi, 35.1% for LB0001, 21.0% for LB0003, 19.9% for LB0004 and 8.6% for BD2630.

Postradiation DSB repair is reduced in LB0003 and LB0004

To ascertain whether this reduced postradiation viability was due to impaired DNA repair, we investigated the induction and rejoining of DNA DSBs using PFGE. Unsynchronized cell cultures were used. Initial DSB induction by 30 Gy ionizing radiation was similar in LB0001, LB0003, LB0004 and Macsi (data not shown). Significantly fewer DSBs were repaired by LB0003 and LB0004 than by Macsi and LB0001 (Figure 2). Almost half (42.9% for LB0001 and 43.7% for Macsi) of DSB observed at 0 min were repaired in 30 min by the control cell lines, whereas only 8.6 and 5.4% were repaired by LB0003 and LB0004. In general, DSB rejoining was complete by 4 h, with normal controls restituting 72–81% of damage and the LB0003 and LB0004 only 35–53%. Even after 24 h, this difference persisted. This suggests that the two cell lines from individuals with late radionecrosis exhibit impaired DSB repair. The reduction in DSB repaired by the fast kinetic pathway is in keeping with previous studies of DSB-rejoining kinetics in DNA-PKcs-deficient cell lines (Allalunis-Turner *et al*., 1995b; DiBiase *et al*., 2000).

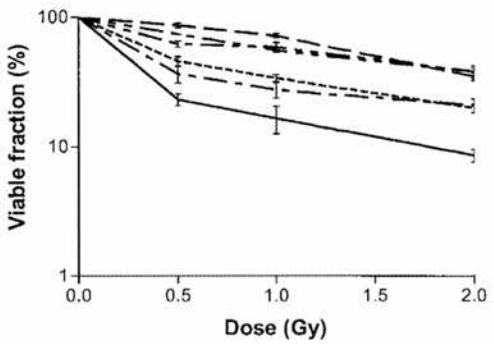

Figure 1 Postradiation viability of cell lines. Data are shown for cell lines Macsi (__..__) Wales (__..__) LB0001 (---) LB0003 (....) and LB0004 (__.__) and BD2630 (____) from an individual with AT. EBV-immortalized lymphoblastoid cells were established by standard techniques (Godsen *et al*., 1986). Control lymphoblastoid cell lines (Macsi and Wales) from individuals with abnormal ocular development, but no history of cancer, were donated by Professor Veronica van Heyningen. BD 2630 was obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were grown in RPMI 1640 medium (Gibco BRL) with 10% FCS, 2 mM L-glutamine, penicillin and streptomycin. Exponentially growing cells were irradiated at 20°C with a 6 MV linear accelerator at 3.2 Gy/min. Cells were plated in triplicate at densities of 5×10^4–5×10^5 cells/ml and incubated at 37°C. A colorimetric proliferation assay using a compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) reduced by metabolically active cells was used to determine viable cells (Promega Cell Titer 96 System). Similar assays have been shown to correlate well with clonogenic assays in estimating radiosensitivity (Price and McMillan, 1990). On days 1, 2, 3, 4 and 5 postirradiation, 20 μl MTS was added to each well and incubated for 4 h. Optical density (OD) measurements were made at 450 nm, and triplicate data averaged after correction for background absorbance. Surviving fractions were estimated by dividing the mean OD of the irradiated sample wells by that of the nonirradiated sample wells from the day 5 assay results, at which time cell lines had returned to exponential growth following radiation-induced cell cycle delay, and before contact inhibition of cell growth within the wells develops. However, unirradiated cells had often ceased exponential growth by day 4, and control data were extrapolated to day 5 to allow calculation of the surviving fraction. This approach has been necessary when using the MTT proliferation assay (Price and McMillan, 1990)

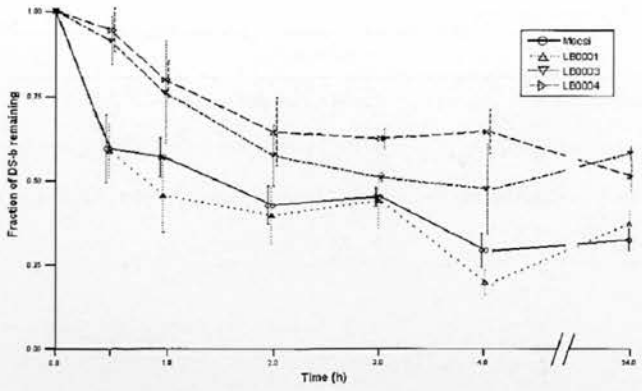

Figure 2 Kinetics of repair of DNA damage measured by PFGE assay. Unsynchronized cells from cell lines Macsi (–○–), LB0001 (···△···), LB0003 (–▽–) and LB0004 (-▷) were counted, aliquoted (4×10^5) and irradiated on ice with 30 Gy using a caesium source (Gammacell 40 Exactor) at 1.06 Gy/min. Following irradiation, cells were incubated with fresh RPMI in 5% CO_2 at 37°C. Unirradiated cells and cells at each time point were harvested and washed. Each pellet was immediately resuspended in 0.5% Sea Plaque low melting temperature agarose at 38°C. The mixture was pipetted into prewarmed 75 μl plug moulds (Bio-Rad) and allowed to set at 4°C. Cell plugs were transferred to 50 ml tubes containing three volumes of ice cold digestion cocktail (1% *N*-lauroylsarcosine (Sigma), 0.1 mg/ml Proteinase K (Sigma), 0.1 M EDTA, 0.01 M Tris-HCl, 0.02 M NaCl) and incubated for 1 h at 4°C, 2 h at 50°C and in two volumes of the digestion cocktail containing 0.1 mg/ml Proteinase K for 18 h at 50°C. Plugs were washed at room temperature in 50 volumes of Tris-EDTA (pH 7.6) with five changes of buffer over 3 h, in two volumes of Tris-EDTA (pH 7.6) containing 40 μg/ml phenylmethylsulphonylfluoride for 30 min at 50°C, and in 50 volumes of Tris-EDTA (pH 7.6) with three changes of buffer and stored in the same solution at 4°C. Cell plugs were cut into 25 μl samples and loaded into the wells of a 0.7% agarose (Seakem Gold agarose, BMA) gel prepared in 0.5 × TBE (45 mM Tris, 1 mM EDTA, 45 mM boric acid, pH 8.5). A reference *Saccharomyces cerevisiae* chromosome molecular weight marker was added to each gel. The wells were sealed with 0.7% LMP agarose. The PFGE unit (Bio-Rad CHEF-DRII) was filled with 2 l of 0.5 × TBE. The sample (at 10°C) was run at 45 V (1.7 V/cm) with a pulse time of 75 min for 40 h, followed by 100 V, 30 min pulse time, for 2 h. Following electrophoresis, the gel was dried, rinsed with double distilled water and placed in 150 ml SYBR Green I (BMA) (1 : 10 000) in 0.5 TBE pH 8.0 at 50°C for 3 h, cooled for 10 min on ice and analysed using a SynGene Gel Documentation System with a Syb-100 SYBR green filter. Gel images were analysed using the Gene Tools software (SynGene). The residual damage fraction was calculated as the ratio of the intensity of the material entering the gel lane to the total intensity of plug plus this material. All data were corrected by subtraction of the nonspecific migration detected in unirradiated control samples. The DSB remaining at a given time of postirradiation cell incubation was normalized to the incidence of DSB at time zero set at 100%. Three independent experiments were carried out for each cell line with two replicates per experiment

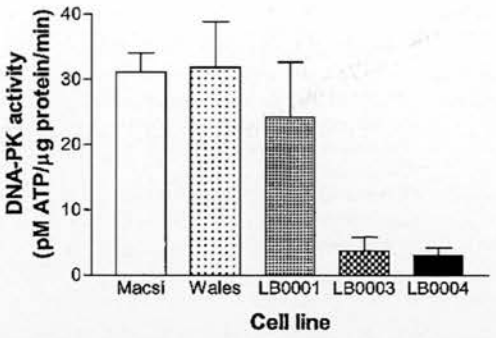

Figure 3 DNA-PK activity in cell lines. Exponentially growing cells from the cell lines Macsi, Wales, LB0001, LB0003 and LB0004 were harvested, and whole-cell extracts prepared (Manley *et al*., 1983). Briefly, 0.8–1 l of cells at a density of 0.5–1.0 × 10^6 cells/ml were pelleted and washed. The pellet was resuspended in four packed cell volumes (PCV) hypotonic lysis buffer (10 mM Tris-HCl pH 8, 1 mM EDTA, 5 mM DTT and protease inhibitors) and left on ice for 20 min. The cells were disrupted in a Dounce homogenizer, and an additional 4 PCV 50 mM Tris-HCl pH8, 10 mM $MgCl_2$, 2 mM DTT, 25% sucrose and 50% glycerol added, followed by one PCV ammonium sulphate. After 30 min, the extract was centrifuged at 38 000 r.p.m. for 3 h at 4°C. The supernatant was removed, and 0.33 g/ml ammonium sulphate added. The extract was centrifuged at 10 000 *g* for 30 min, and the precipitate recovered and resuspended in buffer containing 25 mM HEPES pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 1 mM EDTA and 17% glycerol. This was dialysed against two changes of the same buffer for 6 h, centrifuged at 10 000 *g* for 10 min, and the supernatant snap frozen and stored at −70°C. DNA-PK activity was assayed by measuring the phosphorylation of a biotinylated p53-derived peptide substrate (Promega SignaTECT DNA-Dependent Protein Kinase (DNA-PK) Assay System) by cell extracts in the presence or absence of calf-thymus DNA. Incorporation of γ-[^{32}P] into the peptide was analysed with a phosphorimager (confirmed by scintillation counting) and DNA-PK activity calculated with a correction for background kinase activity. Results were based on at least three experiments using extracts prepared on at least two separate occasions

DNA-PK activity is reduced in cell-free extracts from LB0003 and LB0004

The DNA-PK complex plays a key role in NHEJ. We hypothesized that DNA-PK kinase activity might be one of the determining factors in DSB repair, and a surrogate for the major repair activity in these experiments, where the majority of cells were in G_1 or early S phase. We assayed DNA-PK activity in the cell lines LB0003 and LB0004 from patients with late morbidity, LB0001 from an acute over-reactor, and Macsi and Wales from individuals without cancer. To assay DNA-PK activity, DNA containing termini and all three complex subunits are required. Phosphorylation of a DNA-PK peptide substrate was measured in the presence of calf thymus DNA and corrected for background phosphorylation (in the absence of calf thymus DNA) by nonspecific kinases. We observed a 6–10-fold reduction in DNA-PK activity in the cell lines LB0003 and LB0004 compared to LB0001, Macsi and Wales ($P<0.0001$ for LB0003 and LB0004 *vs* Macsi; $P<0.0005$ for LB0003 and LB0004 *vs* Wales; and $P<0.002$ for LB0003 and LB0004 *vs* LB0001) (Figure 3).

To confirm that other DNA damage and signalling pathways, including ATM which may also be stimulated by DNA strand breaks were intact (and hence the reduction in protein kinase activity in the presence of DNA ends was due to reduced DNA-PK activity), we measured the levels of p53 after 4 Gy ionizing radiation to confirm that normal upregulation of the protein occurred after radiation of these cell lines. This was normal in all cell lines tested, indicating normal ATM activity in these cell lines (data not shown).

A correlation between DSB rejoining, radiosensitivity and DNA-PK activity has been reported in lung cancer (Sirzen *et al*., 1999) and squamous cell cancer cell lines (Polischouk *et al*., 1999), and between radiosensitivity and DNA-PK activity in oesophageal cancer cell lines (Zhao *et al*., 2000). In contrast, one group reported no correlation between DNA-PK activity and radiosensitivity in glioma cell lines (Allalunis-Turner *et al*., 1995a). This may indicate that other factors have more effect on radiosensitivity in gliomas, which are highly radioresistant.

The reduced DNA-PK activity we observed in EBV-immortalized cell lines does not seem to be an artefact of transformation or of previous radiotherapy. Normal levels of activity were seen in all the control EBV-transformed cell lines, one of which (LB0001) was derived from a patient who had received radiotherapy over 2 years previously. This strongly suggests that the association of reduced DNA-PK activity with reduced postradiation viability in these cell lines is a reflection of an underlying DNA repair deficiency *in vivo*, and may have contributed to the injury in these patients. Another group has used EBV-transformed cells to investigate radiosensitivity, and has also shown an association between increased cellular radiosensitivity and the risk of late injury (Alapetite *et al*., 1999).

All DNA-PK subunits are present in cell-free extracts from LB0003 and LB0004

The residual DNA-PK activity observed in the cell lines LB0003 and LB0004 suggested that these cell lines were unlikely to be totally deficient in DNA-PKcs, Ku70 or Ku80. To confirm this, we performed immunoblots against these proteins in the cell lines with reduced DNA-PK activity, LB0003 and LB0004, and controls (LB0001, Macsi and Wales). We could not detect a significant difference in the level of Ku 80 and 70 proteins among any of the cell lines (Figure 4a and b). DNA-PKcs was present in both the cell lines with reduced activity and the control cell lines (Figure 4c).

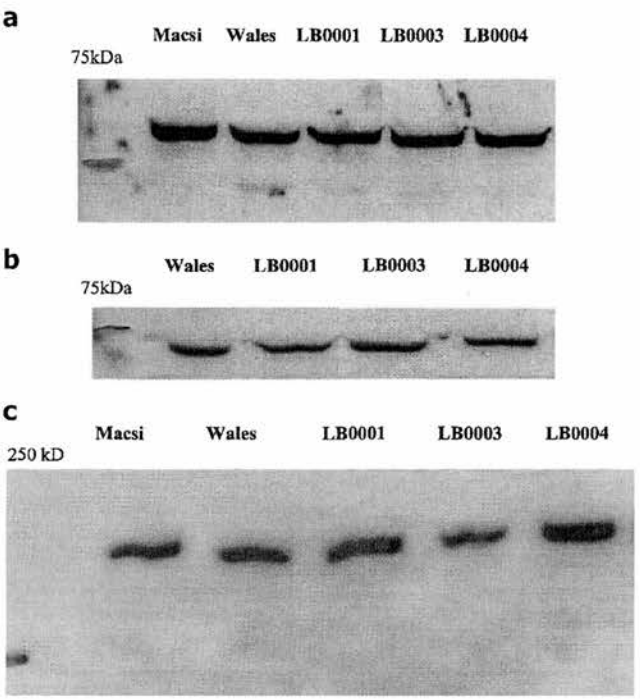

Figure 4 (**a-c**) Immunoblot of subunits of DNA-PK in indicated cell lines. Exponentially growing cells from cell lines Macsi, Wales, LB0001, LB0003 and LB0004, were lysed in 50 mM Tris pH 7.5, 1 mM EDTA, 10 mM DTT, 0.2% Triton X-100 and a protease inhibitor cocktail (Sigma). Aliquots (20 μl) of each sample containing equivalent amounts of protein were separated by SDS–PAGE. Following transfer to nitrocellulose membrane (Amersham), Ponceau's staining was used to confirm protein loading and transfer. After blocking with 5% milk, the membranes were probed with (**a**) anti-Ku80 (Serotec AHP318), (**b**) anti-Ku 70 (Serotec AHP316) and (**c**) anti-DNA-PKcs (Ab-2, Neomarkers, raised against the N-terminus of the protein). Detection was performed using ECL (Amersham). The immunoblots were performed at least twice using separate extracts in each case. The marker was from Amersham (RPN 800)

Conclusion

No humans with a DNA-PKcs or Ku null phenotype have been described. While the two cell lines described here exhibit substantially reduced DNA-PK activity, both have some residual function and express all three protein components of DNA-PK. It is possible that this reduced level of activity is sufficient for function under normal conditions, but insufficient to deal with the extent of DNA repair required following radiotherapy.

Mutations described in transgenic mice and cell lines have shown protein truncations in one of the DNA-PK components, impaired protein-to-protein interactions between the DNA-PK subunits and reduced kinase activity. Our data exclude the former possibility, since we have observed normal size product of all three proteins in immunoblots. However, we cannot, on the current evidence, exclude mutations within the genes encoding one of the three DNA-PK subunits, affecting either protein–protein interactions, DNA-binding or the kinase activity itself, or in genes encoding other unidentified proteins, which interact with or post-translationally modify one of the subunits of DNA-PK. One cell line in the XRCC7 complementation group (XR-C2) has been described with normal levels of DNA-PKcs, but no detectable kinase activity. This cell line exhibited significantly increased radiosensitivity and impaired DSB repair. Sequence analysis of the PRKDC gene in XR-C2 demonstrated a point mutation producing a single amino-acid substitution six residues from the C-terminus of the DNA-PKcs protein (Woods *et al*., 2002), but sequencing of the cell lines reported here has not shown a similar mutation.

This is the first report of a biochemical abnormality in cell lines derived from patients with late radionecrosis at conventional doses; that this occurs in two of the five patients investigated in a pathway so central to recovery from radiation damage in human cells raises the possibility that mild deficiencies in the dominant pathway of DSB repair, perhaps related to single nucleotide

polymorphisms in the DNA-PK genes, might be a common cause of late radionecrosis.

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